

University of Dundee

DOCTOR OF PHILOSOPHY

Recurrent Hypoglycaemia-examining Impact and Strategy

George, Priya

Award date:
2015

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

RECURRENT HYPOGLYCAEMIA- examining IMPACT and STRATEGY

Thesis submitted for the degree of D.Phil,
School of Medicine, University of Dundee

By

Priya George, MB ChB (Manchester), MRCOphth (UK), MRCP (UK)

July 2015

Cardiovascular and Diabetes Medicine
Medical Research Institute
Ninewells Hospital and Medical School
Dundee, DD1 9SY
Scotland

Table of Contents

| | |
|---|----|
| Table of Contents | 2 |
| List of Tables | 8 |
| List of Figures | 10 |
| List of Appendices (in the attached disc) | 12 |
| Index of Abbreviations | 13 |
| Dedication | 19 |
| Acknowledgements..... | 20 |
| Declaration..... | 22 |
| Summary | 23 |
| Introduction | 26 |
| CHAPTER 1 | 26 |
| Definition of hypoglycaemia | 28 |
| Frequency | 32 |
| Mild hypoglycaemia | 32 |
| Severe hypoglycaemia | 34 |
| Risk factors..... | 35 |
| The normal counter-regulatory response in hypoglycaemia..... | 38 |
| Counter-regulatory responses in those with T1D..... | 43 |
| Mechanisms of counter-regulatory failure | 48 |
| Thresholds..... | 54 |
| Impact of defective counter regulation | 56 |
| Impaired Awareness of Hypoglycaemia..... | 56 |
| Effect on cognitive ability..... | 59 |
| Effect on the Hypothalamo-pituitary axis (HPA)..... | 60 |
| CHAPTER 2 | 62 |
| Therapeutic strategies employed to improve Impaired Awareness Hypoglycaemia | 62 |
| Problems with current Insulin preparations | 62 |
| Current treatment strategies..... | 64 |
| Non-drug strategies for reducing hypoglycaemia | 65 |
| Education | 65 |
| Technology..... | 67 |
| Transplantation..... | 73 |
| Drug strategies for reducing hypoglycaemia | 74 |

| | |
|---|-----|
| Approaches to address α cell defects | 74 |
| Approaches to address central defects | 75 |
| Manipulation of sensing mechanisms | 77 |
| Role for dietary supplementation..... | 78 |
| Modulation of the renal haemodynamic..... | 80 |
| Modulating K_{ATP} channels in glucose sensing circuitry | 81 |
| Incretins | 81 |
| CHAPTER 3 | 83 |
| STUDY 1 Use of K_{ATP} channel openers in Acute Hypoglycaemia | 83 |
| Background | 83 |
| Where is glucose sensed? | 83 |
| K_{ATP} channels | 91 |
| Diazoxide; a K_{ATP} channel opener | 96 |
| Pharmacology | 96 |
| Trials of safety and efficacy..... | 98 |
| Methodology..... | 101 |
| Real time Continuous glucose monitoring (CGM) as a tool for avoiding hypoglycaemia prior to clamp studies | 102 |
| Assessment of Impaired Awareness of Hypoglycaemia | 103 |
| Experimental stepped hyperinsulinemic hypoglycemic clamp..... | 105 |
| Physiological measurements | 109 |
| Measurement of sympathetic nervous system | 109 |
| Laboratory assays..... | 110 |
| Symptom assessment | 111 |
| Cognitive function tests. | 112 |
| Data and statistical analysis. | 114 |
| Results..... | 115 |
| Participant characteristics..... | 115 |
| Hyperinsulinemic hypoglycemic clamp studies. | 117 |
| Primary outcome | 118 |
| Secondary outcomes..... | 121 |
| Genetic analysis- E23K polymorphism | 121 |
| Discussion..... | 125 |
| CHAPTER 4 | 133 |
| Glucose variability..... | 134 |
| Definition | 134 |

| | |
|--|-----|
| Why does it occur in Type 1 Diabetes? | 135 |
| Measuring Glucose variability..... | 135 |
| Standard Deviation | 137 |
| J index | 137 |
| Mean absolute glucose | 138 |
| Mean Amplitude of Glycaemic Excursions (MAGE) | 138 |
| Mean of the daily differences (MODD)..... | 139 |
| M value of Schlichtkrull: 1965..... | 139 |
| Continuous overall net glycaemic action (CONGA-n) | 140 |
| Low Blood Glucose Index and High Blood Glucose Index | 140 |
| Glycaemic risk assessment diabetes equation (GRADE)..... | 141 |
| Average Daily Risk Range | 142 |
| Technicalities of measuring GV..... | 143 |
| Associations between GV indices | 144 |
| Is it important?..... | 144 |
| Preclinical studies..... | 147 |
| Effect of GV on the vasculature | 148 |
| Effect of GV on Diabetes related complications | 149 |
| Effect of GV/recurrent hypoglycaemia on the Hypothalamo-pituitary-adrenal axis (HPA) | 151 |
| Role of Glucocorticoids (GC) | 152 |
| Normal cortisol patterns..... | 153 |
| Components of the normal diurnal pattern | 154 |
| Morning rise- Cortisol Awakening Response (CAR) | 154 |
| Slope..... | 157 |
| Area under the curve (AUC) – cortisol | 159 |
| Factors that may affect diurnal patterns | 160 |
| Age | 160 |
| Sex..... | 161 |
| Smoking..... | 161 |
| Oestrogen..... | 161 |
| Obesity | 161 |
| Exercise | 162 |
| Sleep deprivation | 162 |
| Abnormal patterns..... | 162 |
| Other factors affecting the pattern | 165 |
| Time since onset of stress..... | 165 |

| | |
|---|-----|
| Nature of stress..... | 166 |
| Controllability of stress | 166 |
| Emotions elicited by stress | 167 |
| Individual psychiatric sequele | 167 |
| Perceived distress | 167 |
| Early life experiences | 167 |
| Regulation of the HPA axis..... | 168 |
| Dysregulation of the HPA axis..... | 171 |
| Potential impact of dysregulation of the HPA axis on end-organs | 174 |
| Impact of glucose on the HPA axis..... | 175 |
| CHAPTER 5 | 178 |
| STUDY 2; Does adjunct DPP-4inhibitors in patients with T1D improve glucose variability, hypoglycaemic responses and glycaemic control? | 178 |
| Background | 178 |
| Abnormalities of the α cell..... | 179 |
| DPP-4 inhibitors (DPP4-i); their potential utility in hypoglycaemia..... | 180 |
| Trials using DPP4-I in Type 1 Diabetes | 183 |
| A more potent drug; GLP-1R agonists | 186 |
| Non-glucose effects of incretins ; effect on vasculature | 189 |
| Methods..... | 191 |
| Single step hyperinsulinemic hypoglycaemic clamp..... | 195 |
| Blood sampling and analyses | 196 |
| Symptoms and cognitive function tests..... | 196 |
| Statistical analysis | 197 |
| Visits structure | 197 |
| Results..... | 199 |
| Glycaemic control, hypoglycaemia frequency, hypoglycaemia awareness and body weight.... | 201 |
| Glucose variability..... | 203 |
| Adverse events..... | 206 |
| Discussion..... | 208 |
| CHAPTER 6; | 212 |
| STUDY 3; THE GV COHORT; Glycaemic variability and the cortisol curve | 212 |
| Introduction | 212 |
| Methods..... | 214 |
| Assessment of GV - Continuous glucose monitoring..... | 215 |
| Accuracy..... | 216 |

| | |
|---|-----|
| Time lag..... | 216 |
| Problems at hypoglycaemia range..... | 216 |
| Assessment of HPA axis | 217 |
| Saliva | 217 |
| Recruitment | 222 |
| Inclusion criteria..... | 224 |
| Exclusion criteria | 225 |
| Study visits | 225 |
| Results..... | 227 |
| Reliability of this data set..... | 228 |
| Cleaning data | 230 |
| Area under the curve (AUC)..... | 231 |
| Mean levels | 237 |
| Slope..... | 238 |
| CAR..... | 240 |
| Discussion..... | 246 |
| Limitations | 249 |
| Other exploratory studies on this dataset | 250 |
| Correlation between HbA1c and GV parameters | 250 |
| Correlation between GV and insulin doses..... | 253 |
| CHAPTER 7: | 255 |
| STUDY 4; CRH testing in Type 1 Diabetes | 255 |
| Background | 255 |
| CRH stimulation tests..... | 258 |
| Studies so far in those with Type 1 Diabetes..... | 258 |
| Hypothesis for dysregulation of the HPA axis..... | 259 |
| Hypoglycaemia and the HPA axis..... | 262 |
| Methods..... | 263 |
| Results..... | 264 |
| Outliers..... | 264 |
| ACTH parameters | 265 |
| Cortisol parameters | 268 |
| Discussion..... | 271 |
| CHAPTER 7; Conclusions and opportunities for further research | 276 |
| Original papers and abstracts | 284 |
| Reviews | 284 |

| | |
|------------------|-----|
| Prizes | 284 |
| References | 285 |

List of Tables

| | |
|---|-----|
| Table 1; Summary of criteria to enable definition of hypoglycaemia | 29 |
| Table 2; Listing of modifiable and non-modifiable risk factors for further hypoglycaemia, with likely mechanisms. | 35 |
| Table 3; Summary of main studies showing the effect of duration of antecedent hypoglycaemia on subsequent counter-regulatory responses..... | 46 |
| Table 4 Glucose thresholds for the key counter-regulatory hormones and symptoms for the Diazoxide study..... | 121 |
| .Table 5 CAR has been measured in a variety of ways in published studies | 157 |
| Table 6 Summary of trial data of DPP-4 i use in T1D cohorts | 185 |
| Table 7 Inclusion and exclusion criteria for the DPP-4 i study..... | 192 |
| Table 8; Baseline clinical and biochemical characteristics for STUDY 2 | 201 |
| Table 9 Measures of glycemic control and glucose variability following 12-weeks adjunct therapy | 202 |
| Table 10; Baseline characteristics of the original cortisol cohort. N=50 in each group. | 227 |
| Table 11; Individual data points which were excluded from the main data set..... | 230 |
| Table 12 No of missing first values in each group | 231 |
| Table 13; No of subjects in each group with either 3 or 4 missing points..... | 232 |
| Table 14; Table correlating the no of subjects with severe hypoglycaemia and their LBGI scores.... | 234 |
| Table 15; Total number of subjects (excluding single pt with discrepancy between CGM and hypo frequency) analysed after removing those with 3 or 4 points | 234 |
| Table 16; Total number of subjects analysed after removing subjects with 2 or more individual readings missing..... | 236 |
| Table 17; Mean cortisol levels (standard error of the mean) of the 2 main periods of the day. | 238 |
| Table 18; Total number of subjects analysed after removing those subjects with Missing F45 (45 minutes after waking) or F3 (3 hours after waking) and also removing those subjects with 3 or 4 data points missing and also the one subject with the CGM which was not reflective of previous hypo exposure due to treatment regime change. | 239 |
| Table 19; Total number of subjects analysed after removal of subjects with missing points in F0, F30 and F45 (on waking, 30 minutes and 45 minutes after waking) and after removing those with 3 or 4 points missing + removing the 1 subject where CGM did not reflect previous hypo frequency, as insulin doses was reduced quite dramatically prior to insertion of CGM due to safety reasons..... | 240 |
| Table 20; Number of subjects analysed after removal of subjects with missing points within F0, F30 or F45 and removal of those where no CAR could be detected (no increase in cortisol levels from waking)..... | 242 |
| Table 21; Total number of subjects used to analyse AUC _g by removing those subjects who had any missing values in the first 3 data points (F0, F30 and F45) and after removing those with 3 or 4 points missing totally + 1 subject where CGM did not reflect previous hypo frequency, as insulin doses were reduced quite dramatically prior to insertion of CGM due to safety reasons..... | 243 |
| Table 22; Correlation coefficients of the glycaemic variability indices to each of the main parameters of the cortisol curve. *denotes p<0.05 and was statistically significant. | 245 |
| Table 23 Correlation coefficients between HbA1c and Glycaemic variability indices..... | 251 |
| Table 24; Correlation coefficients between total insulin doses and measures of Glycaemic variability. | 253 |

| | |
|--|-----|
| Table 25 The final numbers of subjects out of 10 who were studied as part of the CRH stimulation study..... | 265 |
| Table 26; Demographics of each group examined during the CRH stimulation tests. | 265 |
| Table 27 This shows basal and maximal response (peak) and the maximal rise (Δ) in ACTH responses between the 3 groups..... | 266 |
| Table 28; This shows basal and maximal response (peak) and the maximal rise (Δ) in cortisol responses between the 3 groups..... | 269 |

List of Figures

| | |
|---|-----|
| Figure 1; The normal counter-regulatory system in the non-diabetic human..... | 39 |
| Figure 2; The mechanisms behind the diminished counter-regulation and hypoglycaemia unawareness | 48 |
| Figure 3; Glucose thresholds for activation of CRR hormone release, autonomic and neuroglycopenic symptoms and cognitive deterioration in the non-diabetic human. | 55 |
| Figure 4. Proposed model for development of hypoglycaemia associated autonomic dysfunction (HAAF) | 58 |
| Figure 5; Diagram showing the neural network responsible for generation of the CRR system. | 90 |
| Figure 6; A schematic representation of the ATP sensitive potassium channel. | 92 |
| Figure 7; Consort diagram for STUDY 1. | 116 |
| Figure 8 Glucose profiles during stepped hyperinsulinemic hypoglycemic clamp studies... | 117 |
| Figure 9 Diazoxide amplifies catecholaminergic responses during acute hypoglycaemia in long standing Type 1 Diabetes..... | 119 |
| Figure 10 Cognitive function testing comparing Diazoxide to placebo at each of the glucose nadirs..... | 120 |
| Figure 11 Epinephrine (adrenaline) response at the end of each glucose nadir..... | 122 |
| Figure 12 Epinephrine responses depending on genotype..... | 123 |
| Figure 13 Haemodynamic responses during the clamp studies. | 124 |
| Figure 14; Allostatic load..... | 174 |
| Figure 15 ; Proposed mechanism for STUDY 2. | 189 |
| Figure 16 Diagrammatic representation of DPP-4 i study design | 195 |
| Figure 17 Consort diagram for the DPP-4 i study | 200 |
| Figure 18. Effect of saxagliptin on Hypoglycaemia Awareness, Low Blood Glucose Index and Average Daily Risk Range..... | 204 |
| Figure 19 Blood glucose and Glucose Infusion Rate during STUDY 2 clamp studies | 205 |
| Figure 20 Comparison of Epinephrine and Glucagon responses in placebo/saxagliptin clamp studies. | 206 |
| Figure 21 Effect of saxagliptin/placebo on symptoms and cognitive function tests. | 207 |
| Figure 22 Graphs showing impact of missing points on a. low LBGI b. high LBGI c. control | 233 |
| Figure 23; Graphical representation of AUC of mean and confidence intervals for each of the groups..... | 235 |
| Figure 24; Graph charting the salivary cortisol levels throughout the day..... | 237 |
| Figure 25; Box plot of mean increase in cortisol for each of the 3 groups..... | 241 |
| Figure 26; This shows the ACTH response from baseline to after stimulation with CRH. .. | 266 |
| Figure 27; ACTH response following CRH stimulation. | 267 |
| Figure 28; This shows the cortisol response from baseline to after stimulation with CRH. . | 268 |
| Figure 29; Box and whiskers plot showing the scatter of the baseline cortisol levels in the 3 groups..... | 269 |
| Figure 30 Cortisol and ACTH responses following CRH stimulation. | 270 |

| | |
|---|-----|
| Figure 31; Cortisol responses following CRH stimulation in those with Type 1 Diabetes (T1D) compared to non-diabetic control subjects. | 271 |
|---|-----|

List of Appendices (in the attached disc)

Appendix 1; STUDY 1; Protocol

Appendix 2; STUDY 1; Patient information sheet (PIS)

Appendix 3; STUDY 1; Informed consent sheet

Appendix 4; STUDY 1; GP letter

Appendix 5; STUDY 1; Ethics approval letter

Appendix 6; STUDY 2; Protocol

Appendix 7; STUDY 2; Patient information sheet

Appendix 8; STUDY 2; Informed consent sheet

Appendix 9; STUDY 2; GP letter

Appendix 10; STUDY 2; Ethics approval letter

Appendix 11; STUDY 3 and 4; Protocol

Appendix 12; STUDY 3; Patient information sheet for Controls

Appendix 13; STUDY 3; Patient information sheet for Diabetes

Appendix 14; STUDY 3 and 4; Informed consent form for Controls

Appendix 15; STUDY 3 and 4; Informed consent form for Diabetes

Appendix 16; STUDY 3 and 4; GP letter

Appendix 17; STUDY 4; Patient information sheet for Controls

Appendix 18; STUDY 4; Patient information sheet for Diabetes

Index of Abbreviations

| Acronym | Definition |
|------------------|---|
| ACF | amplitude correction factor |
| ACTH | Adrenocorticotrophic Hormone |
| ADA | American Diabetes Association |
| ADAG | A1c Derived Average Glucose |
| ADRR | Average Daily Risk Ratio |
| AE/SAE | Adverse Event/Serious Adverse Event |
| AGEs | Advanced Glycation End-products |
| AgRP | Agouti Related Peptide |
| AICAR | 5-aminoimidazole-4-carboxamide-1- β -d-ribofuranoside |
| AMP | Adenosine 5'-Monophosphate |
| AMPK | AMP-Activated Protein Kinase |
| ANOVA | Analysis Of Variation |
| AP | Action Potential |
| ATP | Adenosine Triphosphate |
| AUC | Area Under The Curve |
| AUC _g | Area Under The Curve in relation to Ground |
| AUC _i | Area Under The Curve in relation to Increase |
| AVP | Arginine Vasopressin |
| BBB | Blood-Brain Barrier |
| BDR | Background Diabetic Retinopathy |
| BG | Blood Glucose |
| BGAT | Blood Glucose Awareness Training |
| BH | Biochemical Hypoglycaemia |
| BMI | Body Mass Index |

| Acronym | Definition |
|---------|---|
| BP | Blood Pressure |
| cAMP | Cyclic Adenosine MonoPhosphate |
| CAR | Cortisol Awakening Response |
| CARDIA | Coronary Artery Risk Development In young Adult study |
| CBG | Corticosteroid Binding Globulin |
| CFTR | Cystic Fibrosis Transmembrane Conductance Regulator |
| CGM | Continuous Glucose monitoring |
| cGMP | Cyclic Guanosine MonoPhosphate |
| CGMS | Continuous Glucose Monitoring System |
| CI | Confidence Intervals |
| CI/PI | Chief Investigator/Principal Investigator |
| CNS | Central Nervous System |
| CONGA | Continuous Overall Net Glycaemic Action |
| CRC | Clinical Research Centre |
| CRF | Corticotropin-Releasing Factor |
| CRH | Corticotropin-Releasing Hormone |
| CRR | Carbon Removal Rate |
| CRRs | CounterRegulatoryResponses |
| CSF | Cerebrospinal Fluid |
| CSII | Continuous Subcutaneous Insulin Infusion |
| CTA | Clinical Trial Authorisation |
| CTIMP | Clinical Trial of Investigational Medicinal Product |
| CVD | Cardiovascular Disease |
| DAFNE | Dose Adjustment for Normal Eating |
| DCCT | Diabetes Control And Complication Trials |

| Acronym | Definition |
|---------|---|
| DDP-4 i | Dipeptidyl peptidase- 4 inhibitor |
| DM | Diabetic Mellitus |
| DNA | Deoxyribonucleic Acid |
| DSUR | Development Safety Update Report |
| EASD | European Association for the Study of Diabetes |
| EasyGV | Easy Glycaemic Variability |
| ECG | Electro Cardiogram |
| EDIC | Epidemiology of Diabetes Intervention and Complications |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EPC | Endothelial Progenitor Cells |
| FDA | Food And Drug Administration |
| FMD | Flow Mediated Dilatation |
| GABA | Gamma-Aminobutyric Acid |
| GCP | Good Clinical Practice |
| GE | Glucose Excited |
| GH | Growth Hormone |
| GI | Glucose Inhibited |
| GIP | Glucose-Dependent Insulinotropic Polypeptide |
| GIR | Glucose Infusion Rate |
| GLP-1 | Glucagon-Like Peptide 1 |
| GR | Glucocorticoid Receptor |
| GS | Glucose Sensing |
| GV | Glucose Variability |
| HBGI | High Blood Glucose Index |
| IAH | Impaired Awareness of Hypoglycaemia |

| Acronym | Definition |
|------------------|---|
| ICAM | Intercellular Adhesion Molecule |
| IDDM | Insulin-Dependent Diabetes Mellitus |
| IQR | InterQuartile Range |
| JAMA | Journal Of The American Medical Association |
| JDRF | Juvenile Diabetes Research Foundation |
| K _{ATP} | ATP Sensitive Potassium Channels |
| KCO | Potassium Channel Opener |
| LBGI | Low Blood Glucose Index |
| LDL | Low Density Lipoprotein |
| LFT | Liver Function Test |
| LI | Lability Index |
| MAG | Mean Absolute Glucose |
| MAGE | Mean Average Glucose Excursion |
| MAP | Mean Arterial Pressure |
| MAPK | Mitogen-Activated Protein Kinase |
| MARD | Mean Absolute Relative Difference |
| MBG | Mean Blood Glucose |
| MCI | Mild Cognitive Impairment |
| MCT | Medium Chain Triglycerides |
| MDI | Multiple Daily Injections |
| MnInc | Mean Increase |
| MODD | Mean of the Daily Difference |
| MRI | Magnetic Resonance Imaging |
| NAD | Nicotinamide Adenine Dinucleotide |
| NHST | NHS Tayside |

| Acronym | Definition |
|---------|--|
| NTS | Nucleus Of The Solitary Tract |
| oCRH | Ovine Corticotrophin Releasing Hormone |
| OR | Odds Ratio |
| PAI | Plasminogen Activator Inhibitor |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PET | Positron Emission Tomography |
| PHQ-9 | Patient Health Questionnaire-9 |
| PIS | Patient Information Sheet |
| POMC | Pro-opiomelanocortin |
| PPG | Post Prandial Glucose |
| PVN | Paraventricular Nucleus |
| QOL | Quality of Life |
| R&D | Research And Development |
| RAS | Renin-Angiotensin System |
| RCT | Randomized Control Trial |
| REM | Rapid Eye Movement |
| RH | Recurrent Hypoglycaemia |
| RNA | Ribonucleic Acid |
| ROS | Reactive Oxygen Species |
| RR | Relative Risk |
| RT-CGM | Real Time- Continuous Glucose Monitoring |
| SAE | Serious Adverse Event |
| SARs | Serious Adverse Reaction |
| SBP/DBP | Systolic Blood Pressure/Diastolic Blood Pressure |
| SCN | Suprachiasmatic Nucleus |

| Acronym | Definition |
|---------|--|
| SD | Std. Deviation |
| SDRN | Scottish Diabetes Research Network |
| SE | Standard Error |
| SEM | Std. Error Of The Mean |
| SGLT2 | Sodium Glucose Co-transporter 2 |
| SH | Severe Hypoglycaemia |
| SHBG | Sex Hormone-Binding Globulin |
| SMBG | Self-Monitoring Blood Glucose |
| SSRI | Selective Serotonin Reuptake Inhibitor |
| T1D | Type 1 Diabetes |
| T2D | Type 2 Diabetes |
| UKPDS | UK Prospective Diabetes Study |
| VCAM-1 | Vascular Cell Adhesion Mol. 1 |
| VEGF | Vascular Endothelial Growth Factor |
| VIP | Vasoactive Intestinal Peptide |
| VMH | VentroMedial Hypothalamus |
| VMN | Ventro Medial Nucleus |
| vWF | Von Willebrand Factor |

Dedication

To my parents

Drs. Prakash and Premi Mathew

To whom I owe everything

And my baby daughter, Charis who was born the day after I
completed my thesis

Her name means and reminds me of the “the grace of God”

Acknowledgements

First and foremost, I would like to acknowledge the support and mentorship of my supervisor, Professor Rory McCrimmon. He introduced me to clinical research, and has been an inspiration and a wise counsel throughout my research, which has ultimately led to the production of this thesis. His extensive knowledge, academic rigor and foresight of the subject and his tireless patience has been invaluable throughout the project. Not only has he been a wise academic counsel, but he has also been a personal mentor to me, and for that I am truly very grateful.

I am also thankful to all the scientists who work with Prof McCrimmon, for opening my eyes to the basic sciences behind glucose sensing and it has been a real privilege to appreciate the translational nature of the various bench to bedside approaches to understanding hypoglycaemia.

I would also like to thank all the staff at the Clinical Research Centre, Ninewells Hospital, particularly Joanne Forbes and Caron Innes, who have worked alongside with me during the conduct of the clamp experiments. Their conduct, efficiency and manner with our patients was truly appreciated.

I would also like to acknowledge with gratitude the help and support of Mrs Gwen Kiddie (who processed the blood samples) and Mrs Lesley MacFarlane (who analysed all the blood samples).

I would like to thank in particular all the NHS Diabetes Specialist Nurses, who often referred patients to me, who they felt would be suitable for my trials, greatly aiding recruitment, and also to Mrs Lesley Grant, who provided dietetic support for STUDY 2. Furthermore, I would like to acknowledge the Scottish Diabetes Research Network (SDRN), who helped conduct

some of the initial patient searches, from the SCI-Diabetes network and in particular, Natalie Smith (SDRN nurse), who stayed behind in the evenings, and who assisted me in the conduct of the CRH studies (STUDY 4).

I would also like to thank my family, my parents in law (Mr George Oommen and Mrs Susie George) and my sister (Dr. Anna Mathew) and sister in law (Mrs Miriam Varughese), for their constant love, support, encouragement and above all prayers.

Finally, I would like to thank my dear husband, Jacob, who has tirelessly supported me through many long evenings and has been a constant source of love, patience and encouragement throughout the conduct and writing of this thesis. He has been my rock.

Above all, God Almighty has given me all that I needed to complete this thesis, and I am grateful for His wisdom, providence and grace upon my life.

Declaration

I hereby declare that I am the author of this thesis, that all references cited have been consulted by me and that I have carried out all the work described in this thesis. The work described in this thesis have not been previously accepted for a higher degree and I have defined the nature and extent of my contribution to the work within the project described in this thesis.

The work contained in this thesis was carried out during my appointment as Clinical Research Fellow in the Medical Research Institute, Division of Diabetes and Cardiovascular medicine, Ninewells Hospital and Medical School, Dundee between August 2011 and July 2014.

Signed..... Date.....

Summary

Hypoglycaemia and fear of hypoglycaemia is considered one of the greatest barriers to patients optimising their metabolic control in Type 1 Diabetes (T1D). Recurrent hypoglycaemia (RH) leads to diminished symptomatic and hormonal (blunted counter-regulatory) responses to further hypoglycaemia, which collectively form a clinical syndrome referred to as Impaired Awareness of Hypoglycaemia (IAH), a condition that affects one in four of all patients with T1D. IAH markedly increases an individual's risk of severe hypoglycaemia (defined as the need for active external assistance for recovery), which has a recognised morbidity and mortality.

Despite improvements in insulin formulations and delivery devices, rates of severe hypoglycaemia have remained static in the last two decades, therefore there remains an urgent clinical need for novel, alternative strategies to prevent severe hypoglycaemia and restore hypoglycaemia awareness.

This thesis contains studies that represent a first attempt to translate basic research in cells and animal models into novel therapies for impaired awareness of hypoglycaemia in human subjects with Type 1 diabetes. The first study was based on the pre-clinical finding that hypothalamic ATP-sensitive K^+ (K_{ATP}) channels are integral to the detection of hypoglycaemia as well as instrumental to mounting a counter-regulatory response to hypoglycaemia. In a randomised, double-blind hyperinsulinaemic hypoglycaemia clamp study, I compared the effect of an oral K_{ATP} channel activator, Diazoxide, on counterregulatory responses to hypoglycaemia in a cohort of subject with type 1 diabetes and impaired hypoglycaemia awareness. I was able to show that diazoxide magnified the counter-regulatory response (particularly adrenaline) in patients with long standing T1D. In addition, a subgroup analysis revealed that participants with E23K polymorphism in the K_{ATP} channel

had a blunted response to oral diazoxide. This study confirmed for the first time in humans that K_{ATP} channels also played an important role in modulating counterregulatory responses to hypoglycaemia and demonstrated the potential utility of K_{ATP} channel activators to improve counterregulatory responses to hypoglycemia in subjects with T1D.

The second approach focused on the paradoxical role in Type 1 diabetes of dysregulated glucagon secretion during fasting and in the post-prandial states, contributing to hyperinsulinaemia, hypoglycaemia and increased glycaemic variability (GV). I postulated that suppression of glucose-induced glucagon secretion in Type 1 diabetes would reduce hyperinsulinaemia, reduce GV and subsequent exposure to hypoglycemia. This in turn, through hypoglycaemia avoidance, would over time lead to improved hypoglycaemia awareness. To test this, we performed a double-blind, 12 week randomized cross over trial, comparing Saxagliptin, a dipeptidyl peptidase 4 inhibitor (DPP4-I) that had been shown to known for its glucagon suppressive action, with placebo in a small cohort of subjects with Type 1 diabetes. However, we found that 12-weeks Saxagliptin therapy failed to have any significant impact on glucose variability indexes in Type 1 diabetes or exposure to hypoglycaemia. Subsequently, I found no differences between Saxagliptin or vehicle on CRR to hypoglycaemia. These findings therefore do not support the use of DPP4-inhibitors as a means of improving hypoglycaemia awareness in T1D.

For the second part of this thesis, we sought to explore the wider impact of recurrent hypoglycaemia on the hypothalamo-pituitary adrenal (HPA) axis. This study arose from the understanding that hypoglycaemia represents a profound stimulation to the HPA axis, with a rise in peripheral glucocorticoid. Repeated HPA axis stimulation leads to (i) suppression of HPA axis activation in response to recurrent exposure to that stressor - this would have the additional effect of reducing the CRR response to hypoglycaemia, (ii) suppress diurnal variation in cortisol circadian rhythm – associated with increased cardiovascular risk as well

as chronic alteration in mood and cognitive state. To examine this I studied T1D subjects with increased glucose variability (GV) (as an index of hypoglycaemia exposure) and measured circadian variation in salivary cortisol levels. In this study, I report that increased GV is associated with a blunted diurnal cortisol diurnal slope, associated with a significant increase in the area under the curve.

As a follow on study to this, I examined the HPA axis in greater detail by conducting a Corticotrophin releasing hormone (CRH) test, to determine the physiological level of the dysregulation seen in those with T1D and recurrent hypoglycaemia. I found no significant difference to the CRH response between those with recurrent and those with infrequent hypoglycaemia.

In conclusion, this thesis has explored two potential therapeutic strategies, based on current understanding of those mechanisms that underlie the development of impaired hypoglycaemia awareness in Type 1 diabetes and reported that a strategy based on KATP channel activators, but not DPP4i, may have therapeutic potential. This would need to be explored in longer term intervention studies. In addition, I have shown that increased GV and hypoglycaemia exposure is associated with blunting of normal cortisol circadian patterns. Future studies will aim to assess the impact of this on long-term cardiovascular function and atherosclerosis in Type 1 diabetes.

Introduction

CHAPTER 1

Iatrogenic hypoglycaemia was first recognised shortly after the introduction of insulin in 1922 (Banting, Best et al. 1922) but was highlighted as a major issue in the clinical management of Type 1 Diabetes (T1D) following the publication of the Diabetes Control and Complications Trial in 1993 (group 1993). This trial was designed to compare intensive insulin therapy (IIT) using multiple daily injections (MDI) or continuous subcutaneous insulin infusion (CSII), with the aim of achieving near-normal glucose control, against conventional insulin therapy (one or two daily injections) in 1,441 volunteers with T1D recruited from 29 medical centres in the United States and Canada between 1983 and 1989, and followed up until 1993. The results were hugely impressive, demonstrating significant benefits of IIT in terms of both incidence and progression of major microvascular end-points, neuropathy, retinopathy and albuminuria (DCCT group 1993). However, it was also noted that severe hypoglycaemia (requiring active third party assistance to aid recovery) was increased by over three-fold in those on IIT, with many subjects reporting multiple episodes of severe hypoglycaemia (DCCT group 1997). Moreover, it was soon recognised that recurrent hypoglycaemia (RH) leads to a down-regulation of both the magnitude and threshold for activation of counterregulatory hormonal and symptomatic responses during subsequent hypoglycaemia; a clinical phenomenon referred to as impaired awareness of hypoglycaemia (IAH) (McCrimmon & Sherwin). IAH affects approximately 25-30% of all individuals with T1D increasing to almost 50% among those with longer duration diabetes (>20years) (Elliott 2011), and markedly increases the risk of severe hypoglycaemia (SH) by nearly six fold (Geddes, Schopman et al. 2008). Based on these findings, Cryer, in his 1994 Banting Lecture to the American diabetes Association, would refer to hypoglycaemia as “the

limiting factor to the management of [Type 1 diabetes]” (Cryer 2002)..Thus an effective strategy to reduce individual and societal burden of the microvascular complications of T1D could only be achieved at the expense of recurring episodes of severe hypoglycaemia.

The publication of the DCCT served as a major stimulus to basic and clinical research in hypoglycaemia. In the ensuing decades we have learnt more about the mechanisms by which hypoglycaemia is detected and how these mechanisms fail over time in T1D leading to the development of IAH. Newer insulin formulations, insulin delivery devices and delivery strategies have been introduced in order to reduce hypoglycaemia risk, and yet despite this the frequency of severe hypoglycaemia remains relatively unchanged in most western health care systems. This means there is a need for novel therapies and strategies that may help to prevent severe hypoglycaemia in T1D, while allowing individuals to maintain near-normal glucose control over the life-time of their disease.

In this thesis, I will explore the potential merits of two different oral agents as adjuncts to insulin therapy that might serve to prevent severe hypoglycaemia and/or restore awareness of hypoglycaemia. The first is a non-selective K_{ATP} channel opener (diazoxide) and the second will be a dipeptidyl peptidase 4 inhibitor (DPPiVi) (saxagliptin). The first approach was chosen following several publications that revealed the importance of the ATP sensitive potassium channel (K_{ATP}) in the detection and generation of counterregulatory responses to hypoglycaemia (Evans, McCrimmon et al. 2004, McCrimmon, Evans et al. 2005). The second drug was chosen because of its dual action to suppress glucose-stimulated glucagon secretion and to reduce the overall dose of exogenous insulin replacement. Taken together, this would be anticipated to reduce glucose fluctuations, and as a consequence, exposure to hypoglycaemia, indirectly leading to improve the counter-regulatory responses to further hypoglycaemia.

The second part of the thesis, will explore the impact of recurrent hypoglycaemia (RH) and/or glucose variability (GV) on the hypothalamo-pituitary adrenal axis (HPA) - the system which largely controls the body's response to stressful stimuli, in order to gain a wider appreciation of possible maladaptive processes on this system and then speculate the impact on wider systems controlled by the HPA axis.

So to begin, I will give an overview of hypoglycaemia, with a discussion around the challenges of defining hypoglycaemia, the enormity of the problem in those with T1D, the normal defence mechanisms against hypoglycaemia (or the counterregulatory (CRR) defence mechanism), and possible mechanisms of failure of this system. One of the main reasons for failure of this system is the recurrent nature of hypoglycaemia and I will then follow the discussion with the potential impact on other systems as a result of recurrent hypoglycaemia (RH).

Definition of hypoglycaemia

One of the earliest definitions of hypoglycaemia was an episode of low glucose meeting the criteria of Whipple's triad that is of; symptoms consistent with hypoglycaemia, low plasma glucose, and a relief of symptoms when the plasma glucose concentration was raised(Whipple and Frantz 1935). Subsequently, there has been debate as to how to define the exact glucose value at which hypoglycaemia occurs. Most clinicians use the cut-off plasma glucose between 3.5 and 3.9 mmol/L.(Frier 2009). Diabetes UK recommend that individuals should not allow their blood glucose to drop below 4.0mmol/L, but this clearly does not define hypoglycaemia(O'Neill 1997). The American Diabetes Association (ADA) have defined their set threshold at <3.9mmol/L(Workgroup on Hypoglycemia 2005) which has also been endorsed by the Food and Drug Administration (FDA) agency. They deemed it important that their definition should include all episodes of an abnormal plasma glucose

that could potentially harm the individual and therefore included other categories, such as relative and probable hypoglycaemia. The ADA have therefore suggested that 3.9 should be an “alert value”. There is however increasing consensus to make the biochemical criteria less than 3.5mmol/L, as it includes the advice given to children (ISPAD 2009). These biochemical definitions, also highlight the importance of differentiating between lower values for target ranges and values designated to represent true hypoglycaemia.

Table 1; Summary of criteria to enable definition of hypoglycaemia

| Cut off (mmol/L) | Organisation |
|------------------|---|
| Less than 4.0 | Diabetes UK |
| <3.9 | ADA/FDA (American Diabetes Association/Food and Drugs Administration) |
| <3.5 | ISPAD (International Society For Paediatric and Adolescent Diabetes |

Also, a further complication in coming to a definition of hypoglycaemia, lies in differences in reporting. Finger pricking measures whole blood glucose whereas the meters are calibrated to plasma glucose values. Plasma glucose is on average 10% higher than whole blood glucose concentrations and therefore, there can be a considerable error range on most home blood glucose monitoring systems (Cengiz and Tamborlane 2009). (Current standard (ISO) is BG>4.2mmol/L to be within 15% of reference range, and BG<4.2mmol/L not to differ from reference range by more than 0.83mmol/L).

The ADA criteria however are all based on plasma glucose, and therefore is often an overestimate of the true value. Most recently however a joint working group (Seaquist, Anderson et al. 2013) between the ADA and the Endocrine society defined hypoglycaemia as “all episodes of an abnormally low plasma glucose concentration that expose the individual to potential harm”. The absence of an exact figure in this definition was the realisation that glycaemic thresholds are dynamic and change with prior glucose exposure. However for the purposes of patients and caregivers, a pragmatic value of <3.9 mmol/L was given, chosen because it is the glycaemic threshold for activation of counterregulatory responses (CRR) in non-diabetic individuals and the upper limit of plasma glucose at which there is a subsequent reduction of CRR during further episodes of hypoglycaemia. However most patients with T1D do not have a glucagon response (McCrimmon and Sherwin 2010) and therefore using a threshold suitable for non-diabetics has been seen as having a questionable relevance in this context.

In summary, there still exists much debate regarding the cut off point for defining hypoglycaemia. Variable definitions used by different authorities, make comparison of research studies particularly challenging. Although a fixed value maybe somewhat artificial, as in real life situations, the ambient glucose environment and glucose control determine variable thresholds for symptom generation leading to recognition and reporting of hypoglycaemia by an individual, it may be necessary for pragmatic and educational reasons.

In order to get some consensus of hypoglycaemia reporting, a workgroup was set up in 2005, and the suggested definitions described below are generally well accepted by trialists and aids in current reporting. (Workgroup on Hypoglycemia 2005)

a. Severe hypoglycaemia (SH)

A neuroglycopenic event, which requires third party assistance in administering carbohydrate, glucagon, or other resuscitative actions, in aiding recovery. Provided there is quick restoration of neurological status following resuscitative measures, a plasma glucose is not crucial. However this definition spans episodes where carbohydrates are offered by a third party and then ingested by the patient, and so is partially dependant on the patient (this may be the case in both young and elderly patients), and also episodes which involve significant neuroglycopenia and collapse requiring parental administration. Theoretically, administration of glucagon to prevent severe hypoglycaemia could also be included in this category. Therefore inclusion of these other scenarios, could potentially overestimate the prevalence of SH.

b. Documented symptomatic hypoglycaemia (Mild)

Typical symptoms of hypoglycaemia + a plasma glucose $\leq 3.9\text{mmol/L}$

c. Asymptomatic hypoglycaemia (Mild)

No symptoms of hypoglycaemia + a plasma glucose $\leq 3.9\text{mmol/L}$. This could be when the patient tests their plasma glucose co-incidentally before a meal or an insulin dose, and finds it lower than expected. The other situation this may arise, is if someone apart from the patient recognises the signs of hypoglycaemia, even though the patient has no subjective awareness. Traditionally, b and c are usually clumped into the mild hypoglycaemia category in clinical research studies.

d. Probable symptomatic hypoglycaemia

Patients experience typical symptoms of hypoglycaemia which were rectified by administration of appropriate carbohydrates with no documented blood glucose. It is recognised, that if self-testing is neither available or possible, the most pragmatic

approach recognises that if symptoms are reversed by appropriate treatment, then corroborative evidence with blood sugar testing is not an absolute requirement to identify hypoglycaemia.

e. Relative/pseudo hypoglycaemia

Especially important in those with poor glycaemic control, who have typical symptoms of hypoglycaemia at a blood glucose of $>3.9\text{mmol/L}$

The above discussion therefore shows the varying problems with defining hypoglycaemia, and may account for the widely quoted ranges of the frequency of hypoglycaemia in different cohorts of patients.

Frequency

Mild hypoglycaemia

On average, those with T1D experience 2 episodes of mild symptomatic hypoglycaemia per week (Pramming, Thorsteinsson et al. 1991, Frier 2009) . However the true frequency of hypoglycaemia is hard to determine unless it is done prospectively in those having mild symptomatic hypoglycaemia. Even then, a study in 66 T1D subjects(Pramming, Thorsteinsson et al. 1990) in the outpatient setting showed that true biochemical hypoglycaemia (BH) was only present in 29% of symptomatic hypoglycaemia and only 16% of true BH was accompanied with symptoms. Often if an episode is deemed to be mild, the patient may even forget to mention it to their physician, and therefore this may also account for substantial under-reporting. This was reported in a qualitative study whereby relatives of those with IAH, commented on poor recollection of hypoglycaemia from their relative during consultations. (Lawton, Rankin et al. 2014). Estimates of asymptomatic hypoglycaemia are also dependant on the intensity of self-monitoring of blood glucose (SMBG).

Continuous glucose monitoring (CGM) has proved to be a useful tool in this setting and particularly important in identifying hypoglycaemia in those who have lost their warning of hypoglycaemia, or indeed those who have frequent nocturnal hypoglycaemia. As an example, a study in a group of well controlled T1D using CGM showed an average of 2.1 episodes over a 24 hour period.(Kubiak, Hermanns et al. 2004), and even those with intact awareness did not have symptoms during 62% of biochemical hypoglycaemia (BH). Studies have shown that CGM, in those with Impaired Awareness of Hypoglycaemia (IAH) can pick up 40-60% of all hypoglycaemic episodes, which would otherwise go unrecognised even in those who do SMBG regularly(Geddes, Schopman et al. 2008). However there is debate as to the accuracy of CGM at lower blood glucose levels or when the rate of change of blood glucose is rapid. Continuous glucose monitoring systems which measure interstitial glucose is calibrated to finger prick (whole blood) which is often calibrated to plasma glucose, and therefore there can be a lag behind plasma glucose particularly during periods of quick flux. Interstitial glucose has been shown to overestimate blood glucose at all levels of hypoglycaemia ($p < 0.001$), with each 1 mmol/L drop in blood glucose associated with a 0.32 mmol/L difference (Choudhary, Lonnen et al. 2011). A recent study comparing CGM (Guardian Real-Time) with laboratory references showed an overall mean absolute relative difference (MARD) of 16.7%, with 94.6% lying in the Clarke error grid zone (A+B), however there was a failure to accurately detect half of all true hypoglycaemic events (sensitivity 37.5%) with a MARD of 38.8% in the hypoglycaemic range(Zijlstra, Heise et al. 2013).

Therefore there are still deficiencies in current technological methods to detect mild hypoglycaemia. Self-reporting is problematic because of poor correlation with symptoms and blood glucose (even in those with intact awareness). Under-reporting particularly of mild symptomatic and asymptomatic hypoglycaemia accounts for 88% of all hypoglycaemic

events(Brod, Christensen et al. 2011) . CGM devices may overcome some of these problems but deficiencies in sensitivity in the hypoglycaemic range may also fail to provide a true measurement of the burden of mild hypoglycaemia(Zijlstra, Heise et al. 2013).

Severe hypoglycaemia

Yearly incidence rates of severe hypoglycaemia (SH) in those with T1D, of up to 42% have been reported in observational studies conducted in both UK and Europe. (ter Braak, Appelman et al. 2000, Pedersen-Bjergaard, Pramming et al. 2004, Gimenez, Lopez et al. 2012). The UK Hypoglycaemic Study Group in those with T1D showed an incidence of severe hypoglycaemia of 1.1 and 3.2 episodes per patient year in those with disease duration of less than 5 years greater than 15 years respectively.

However, the definition of severe hypoglycaemia in some of these studies included those who “needed assistance”. This definition may therefore include those who were given glucagon in the context of mild hypoglycaemia to avoid a more severe episode (uncommon) but also the very young and elderly population due to other reasons. As a result, it has been proposed that it may be more accurate to define severe hypoglycaemia as that which involves a seizure or loss of consciousness (LOC). (Weinstock, Xing et al. 2013). In the US, T1D exchange clinic registry data (26,000 participants from paediatric and adult endocrinology practices) (Weinstock, Xing et al. 2013) whereby this definition of either LOC or seizure was employed, 18.6% and 11.8% had SH (if duration of diabetes >40 years and <40 years respectively). These figures are fewer than previously reported probably reflecting the narrower criteria used in the T1D Exchange clinic network.

Interestingly frequency of SH in RCTs (Little, Chadwick et al. 2012) remains far lower than seen in routine clinical practice (Weinstock, Xing et al. 2013) indicating either that the implementation of strategies employed in these RCTs have not translated as yet to routine

clinical care or that the frequent monitoring and repeated contact with a health care professional during RCTs has a very positive influence on the patient and improves diabetes outcomes compared to routine clinical care.

From the above discussion, it follows that hypoglycaemia is almost inevitable in those with T1D, however there are numerous risk factors for multiple episodes of hypoglycaemia and these are summarised below.

Risk factors

Often one of the first steps in reducing the burden of hypoglycaemia on a patient is through identification of scenarios that confer risk, with a discussion around avoidance or preventative strategies. However, on other occasions, the cause is often multi-factorial, or no definite cause can be identified. There are multiple factors that increase the likelihood of developing hypoglycaemia and these are summarised below.

Table 2; Listing of modifiable and non-modifiable risk factors for further hypoglycaemia, with likely mechanisms.

| Modifiable risk factors | Mechanism | References |
|---|---|--|
| Antecedent hypoglycaemia (AH) | Increased catecholamine and opioid production during AH leading to suppression of CRR subsequently | (Heller and Cryer 1991) (Caprio, Gerety et al. 1991) |
| History of previous severe hypoglycaemia | Blunting of counter-regulatory responses. SH in the previous 6 months was the strongest predictor of further SH in the JDRF-CGM RCT. | (DCCT Hypo Study, 1997) (Donnelly, Morris et al. 2005) (Fiallo-Scharer, Cheng et al. 2011) |
| Impaired awareness of hypoglycaemia (IAH) | IAH RR 6.2 (4.6-8.4) p<0.0001 | (Pedersen-Bjergaard, Pramming et al. 2004) |

| | | |
|---|--|--|
| | Completely Unaware RR 20 (13-31) $p < 0.0001$ | |
| Low insulin requirements ($< 0.43 \text{ u/kg}$) irrespective of BMI were associated with higher rates of SH | Higher insulin sensitivity | (Weinstock, Xing et al. 2013) |
| Glycaemic variability (GV) | Increased GV associated with hypoglycaemia | (Janssen, Snoek et al. 2000) |
| Tight glycaemic control | HbA1c has been shown to have a U shaped relationship with frequency of SH, with the lowest frequency at 53- 58 mmol/mol | (Weinstock, Xing et al. 2013) |
| Exercise | Inability to reduce circulating insulin increases risk. Decreases the CRR to subsequent hypoglycaemia. (Galassetti, Mann et al. 2001) | (Galassetti, Mann et al. 2001) (Milman, Leu et al. 2012) |
| Alcohol | Endogenous hepatic glucose production is reduced. Reduces peripheral autonomic responses Impairs cognitive function but only in fasted patients | (Vindedzis, Marsh et al. 2013) |
| Smoking | Could be indirect effect due to differences in lifestyle. | (Pedersen-Bjergaard, Pramming et al. 2004) |

| | | |
|-----------------------|---|---|
| Peripheral neuropathy | Mechanism not clear Symptomatic; OR 2.2 (1.5-3.3) $p < 0.0001$ Those with and without PN ($p = 0.002$) | (Pedersen-Bjergaard, Pramming et al. 2004) (ter Braak, Appelman et al. 2000) |
| Renal failure | Reduction in insulin clearance. Degradation of insulin in peripheral tissues is decreased. Suboptimal nutrition may mean poor glycogen stores. Reduction in renal gluconeogenesis | (Moen, Zhan et al. 2009) |

Non-modifiable risk factors

| | | |
|-----------------------|--|---|
| No residual C peptide | Indicator of more complete loss of endogenous insulin and associated with greater loss of glucagon responses | DCCT (1993) |
| Duration of diabetes | Appears to be the strongest determinant of frequency of SH | DCCT Hypo study (1997) (Weinstock, Xing et al. 2013) |
| Female sex | Catecholamine levels are suppressed during moderate hypoglycaemia. Peripheral insulin sensitivity appears lower in women, but women have enhanced hepatic sensitivity. It is thought to be mediated by oestrogen | (Amiel et al, 1993, (Sandoval, Ertl et al. 2003) |
| Older age | Despite an intact counter-regulatory response, a small study showed reduced symptomatic awareness, may be due to reduced end organ response to counter-regulatory hormones. | (Brierley, Broughton et al. 1995) |

| | | |
|-------|---|--|
| Sleep | Reduced sympatho-adrenal responses during sleep, which also results in lower likelihood of being awakened during hypoglycaemia. | (Banarer et al, 2003) (Jones et al, 1998) |
| | May be part of a general reduction in overall sympathetic activity seen in non-REM sleep. | |
| | Asymptomatic nocturnal hypo may suppress response to further hypo | |

| | | |
|--------------------------------------|--------------------|-------------------------------|
| Lower education levels (p=0.03) | Observational data | (Weinstock, Xing et al. 2013) |
| Lower household incomes (p<0.001) | | |

Having considered possible risk factors of hypoglycaemia, I will move on to the physiological compensatory mechanisms that occur in response to a reduction in blood sugar in a non-diabetic individual prior to a discussion on how the system fails in those with recurrent hypoglycaemia and Type 1 Diabetes.

The normal counter-regulatory response in hypoglycaemia

A continuous supply of glucose to the brain, is maintained by a set of complex peripheral, central and behavioural defence systems to enable optimal performance (Cryer and Gerich 1985) delivered through a sturdy counter-regulatory system. The key components of the counter-regulatory system were first identified in the early 1980s and will be discussed below (Drost, Gruneklee et al. 1980) and is pictorially displayed in Figure 1.

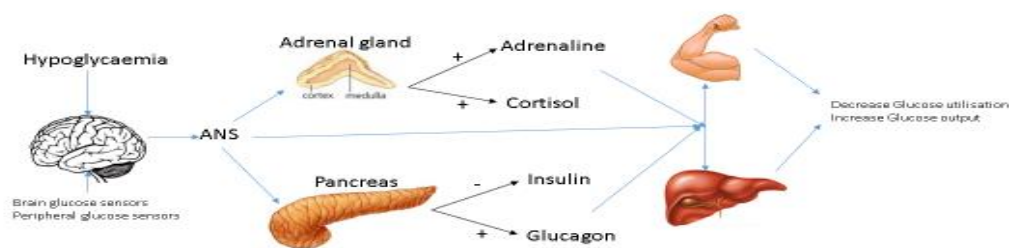


Figure 1; The normal counter-regulatory system in the non-diabetic human.

The main glucose sensors are found in the brain with signals transmitted via the autonomic nervous system to the main effector organs, which ultimately release glucose and decrease glucose utilisation, restoring euglycaemia. ANS Autonomic Nervous System. Adapted from (Martin-Timon and Del Canizo-Gomez 2015).

Firstly, the hormonal systems in place, in the non-diabetic are listed below,

1. Suppression of endogenous insulin secretion. Decrease of insulin produced by the beta cells of the pancreas. In healthy humans, this occurs whilst plasma glucose is still in the physiological range at approximately 4.4mmol/L. (Fanelli, Pampanelli et al. 1994) This decrease in insulin and co-secreted GABA are thought to lift tonic inhibition of the α cell which results in an increased secretion of glucagon (Xu, Kumar et al. 2006). Together with the low levels of portal insulin, this stimulates hepatic glycogenolysis and gluconeogenesis with a resulting increase in hepatic glucose production.
2. Secretion of the α cell product – glucagon. Glucagon is a 29 amino acid peptide, cleaved by pro-hormone convertase-2 from the pro-glucagon molecule in pancreatic islet alpha cells. Secretion of glucagon occurs as the blood glucose drops and the threshold for release is around an arterialized blood glucose of 3.8mmol/L (Fanelli, Pampanelli et al. 1994). This was found to play a primary role in counter-regulation (Gerich, Langlois et al. 1973, Rizza, Cryer et al. 1979), as early studies

using catecholamine deficient patients with spinal cord transections and those who had a previous adrenalectomy were found to be still capable of restoring euglycaemia during induced hypoglycaemia. Activation of the glucagon receptor stimulates a (Gs) protein which increases production of cAMP through activation of adenylate cyclase. Glucagon is directly secreted into the portal vein and acts directly on the G-protein coupled receptors in the liver to enhance glucose production. (Ramnanan, Edgerton et al. 2011). The majority of this effect is through hepatic glycogenolysis. However in combination with adrenaline, which enhances delivery of gluconeogenic precursors, and when substrate availability increases, (during meals) glucagon also stimulates hepatic gluconeogenesis.(Gustavson, Chu et al. 2003). Glucagon also inhibits glycolysis and glycogen synthesis. Glucagon can also stimulate lipolysis through activation of cAMP and lipases. In Kir6.2 $-/-$ deficient mice which lacked both functional K_{ATP} channels and VMH glucose excitatory (GE) (neurons which increase their output as glucose levels increase)neurons, glucagon response was severely impaired, suggesting that there is also a central control of glucagon release(Miki, Liss et al. 2001). In recent studies, a central control of glucagon has been shown to originate from the autonomic nervous system through the para and sympathetic nervous systems and also via adrenal secretion of adrenaline(Porte, Woods et al. 1975). The contribution of each of these systems appears to be related to the severity of the hypoglycaemia. Glucagon secretion also appears to be sustained in isolated islets suggesting other factors involved in its release(Stagner, Samols et al. 1980). There are still contradictory findings as to whether glucose has a direct or indirect effect on the α cells(Gilon 2014). Other factors that have been suggested to regulate glucagon secretion include nutrients, insulin, zinc, GABA, glutamate, somatostatin, ghrelin, and glucagon itself. (Gromada, Franklin et al. 2007).

3. Autonomic responses during acute hypoglycaemia(see figure 1); There is a rise in adrenaline (epinephrine) in the circulation and noradrenaline at the nerve terminals produced by the adrenal medulla(Sprague and Arbelaez 2011).

Adrenaline acts on alpha and beta adrenergic receptors at multiple end organs, and is effectual in maintaining a sustained increase in glucose, by increasing glycogenolysis and gluconeogenesis in the liver(Cryer, Davis et al. 2003). It also acts on the pancreas to reduce insulin and increase glucagon secretion. Peripherally, it reduces glucose uptake and utilisation and increases glycolysis in the muscle, and increases lipolysis in the adipose tissue. (Cryer 2002) Neurogenic symptoms occur as a result of the activation of the autonomic nervous system. Presence of these symptoms in patients' who have had an adrenalectomy, shows these symptoms are generated through sympathetic neuronal activation rather than through adrenaline release from the adrenal medulla whilst the noradrenaline responses and the haemodynamic responses are mainly due to adreno-medullary release.(DeRosa and Cryer 2004)

Increased adrenaline also accounts for the increased cardiac work load, and promotes ECG changes such as QT lengthening, which can provoke arrhythmias particularly in a patients with coronary artery disease(Frier, Schernthaner et al. 2011).

4. Growth hormone; Secretion of growth hormone (GH) usually occurs at a glucose threshold of 3.7mmol/L. However studies which rendered subjects selectively growth hormone deficient during hypoglycaemia, showed no difference in glucose appearance and disappearance compared to control subjects(Rizza, Cryer et al. 1979), therefore it is postulated that GH does not have any role in the immediate recovery but may be more important in more prolonged hypoglycaemia.
5. Glucocorticoid; similarly cortisol is found to also have a role during more prolonged hypoglycaemia, with a glucose threshold for release at approximately 3.2mmol/L.

Studies have suggested that this lower threshold for HPA activation is not true, but due to a delay in activation of ACTH which may require a longer set period of hypoglycaemia (Schwartz, Clutter et al. 1987). Both GH and cortisol stimulate lipolysis in adipose tissue, ketogenesis and gluconeogenesis in the liver. When GH and cortisol were both blocked, it has been shown that glucose recovery was much slower despite increases in adrenaline.(De Feo, Perriello et al. 1989)

6. Symptom profile; alongside the above, autonomic symptoms arising from the sympathetic neural response alert the individual of progressive hypoglycaemia. These symptoms are key to recognition of hypoglycaemia. These symptoms have been shown to be broadly divided into a three factor model (autonomic, neuroglycopenic and non-specific) (Deary, Hepburn et al. 1993). Neuroendocrine responses are responsible for generation of the autonomic component, whereas neuroglycopenic symptoms are thought to be as a result of brain cortical glucopenia(Hepburn 1993). Mitrakou et al showed that the glycaemic thresholds for the autonomic symptoms (3.2mmol/L) were higher than that for neuroglycopenic symptoms (2.8mmol/L) (Mitrakou, Ryan et al. 1991). Symptoms can be highly variable in patients and depends on a number of factors such as the exposure to hypoglycaemia in the preceding 48 hours, rate of glucose decline, the person's current level of activity, duration of diabetes and prevailing glycaemic control. Therefore, a patient who has poor glycaemic control may have symptoms even when the blood sugar is much above 4.0mmol/L (Boyle, Schwartz et al. 1988) termed by the ADA as relative hypoglycaemia and vice versa, with a patient with strict glycaemic control only having symptoms at a very low glucose level(Boyle, Kempers et al. 1995). However, if they do have symptoms, patients tend to have similar patterns, albeit atypical.

7. Cognitive function

Generally in a non-diabetic individual, cognition is impaired when plasma glucose drops below 2.9mmol/L. The most impaired domains of cognition tend to be information processing, attention, memory and reaction times(McCrimmon, Ryan et al. 2012). It is often the most complex processes that are affected by acute hypoglycaemia, and therefore it has been emphasised that it is crucial to use a battery of psychometric tests to detect a subtle cognitive defect(Heller and Macdonald 1996). Those however who experience RH, appear to have cognitive preservation, even when the blood sugars are extremely low, and this has been seen to be part of the adaptive processes of hypoglycaemia. (Fanelli, Pampanelli et al. 1994)

Therefore, through these above hormonal and symptomatic responses, blood glucose is kept in a very tight physiological range, ensuring optimal delivery of glucose to vital organs, particularly the brain. The following discussion describes when these mechanisms start to fail in T1D, as the islet cells are destroyed and glucagon release becomes dysregulated.

Counter-regulatory responses in those with T1D

Major defects in the generation of counter-regulatory response contribute to the high frequency of hypoglycaemia seen in those with T1D.

Starting at 1-8 months after diagnosis(Siafarikas, Johnston et al. 2012) and certainly within 5 years, almost all individuals fail to generate an adequate glucagon response to hypoglycaemia and is a key feature of impaired hypoglycaemia counter-regulation. (McCrimmon 2008) The portal insulin: glucagon ratio is primary in controlling hepatic glucose production. An inadequate glucagon response is associated with marked delay in glucose recovery as a result

of reduced glycogenolysis and gluconeogenesis. (Gerich, Langlois et al. 1973). Several intra and extra pancreatic defects have been identified which may explain this.

a. Intra -islet factors

- i. Failure of local regulation on the α -cell signalling by insulin, zinc and/or GABA(Fukuda, Tanaka et al. 1988). This is suggested because there is a loss of this stimulus specific (i.e. hypoglycaemia) glucagon secretion which closely follows the decrease in C-peptide levels (Fukuda, Tanaka et al. 1988).
- ii. Elevated somatostatin within the islets has also been shown to suppress the glucagon response to hypoglycaemia. This was illustrated in rodent models when glucagon responses could be restored when there was pharmacological antagonism of the somatostatin receptor. (Yue, Burdett et al. 2012)
- iii. There is now evidence suggesting there is a local intra-islet sympathetic neuropathy. (Munding, Mei et al. 2003)

b. Extra-islet factors

- i. The VMH has been recently been shown to modulate the interaction between insulin and glucagon.(Paranjape, Chan et al. 2010). Recurrent hypoglycaemia has shown to significantly suppress the VMH stimulation of the- α cells. (Borg, Borg et al. 1999)

2. Reduced autonomic stimulation-

- a. Attenuated adrenaline release; within 10 years of diagnosis, the majority of those with T1D have a reduced autonomic response (Fanelli, Pampanelli et al. 1997). The reduction in adrenaline during subsequent hypoglycaemia, is as a result of a resetting of the glycaemic threshold for activation at a lower level,

and this may be due to both the duration of diabetes as well as exposure to hypoglycaemia, rather than a reduced secretory capacity. It has been shown that a single episode of antecedent hypoglycaemia is sufficient to attenuate the CRR to further hypoglycaemia (Heller and Cryer 1991, Davis, Mellman et al. 1992). The magnitude of attenuation appears to be linked to the depth, duration and frequency of the hypoglycaemia stimulus. This reduction is thought to be as a result of reduced sympathetic drive to the adrenals rather than a defective secretory capacity of the adrenals. There is also a reduced magnitude of adrenaline release with age irrespective of diabetes, with a study showing that children with/without (Amiel, Simonson et al. 1987) T1D have almost twice as much adrenaline as adults. The elderly in addition have been shown to have a reduced sensitivity to catecholamines (Pende, Musso et al. 1991).

One of the original studies looking at the mechanisms of this phenomenon showed that intravenous administration of adrenergic blockers could prevent development of counter-regulatory failure the subsequent day during hypoglycaemia, suggesting it is the generation of the adrenergic response during hypoglycaemia that potentiates the defective counter-regulation. (Ramanathan and Cryer 2011).

Table 3; Summary of main studies showing the effect of duration of antecedent hypoglycaemia on subsequent counter-regulatory responses.

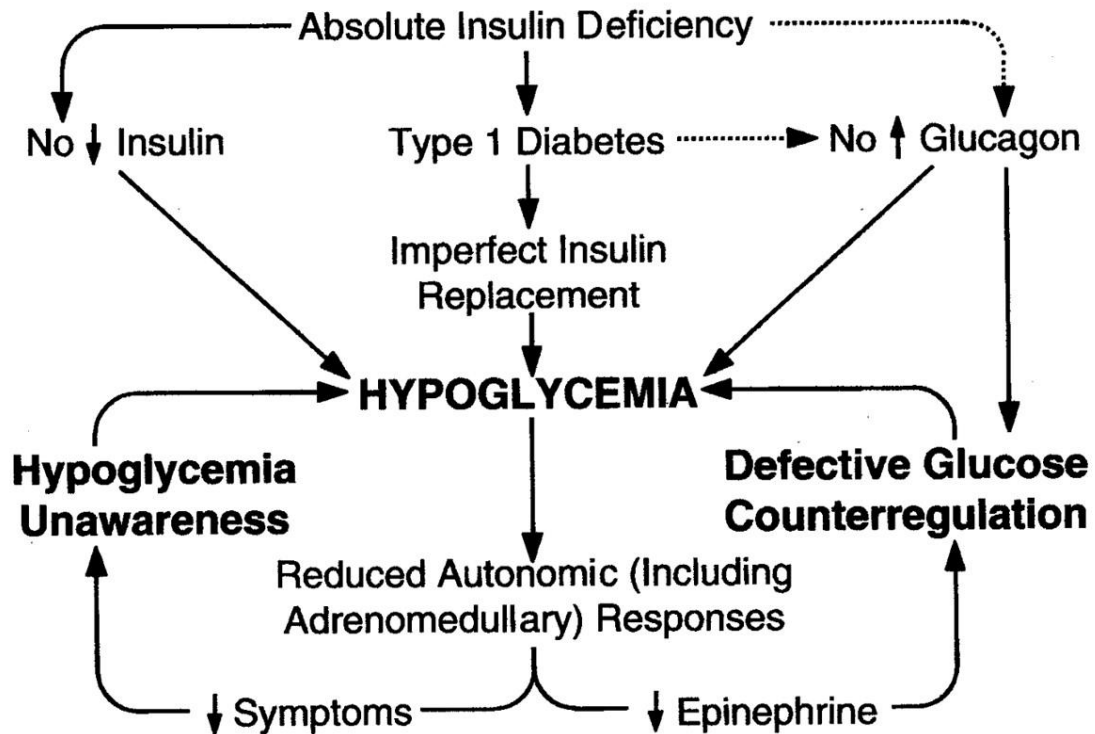
| Stimulus | Effect | |
|---|---|-------------------------------|
| 1 hypo-30mins (2.8mmol/L) on day 1 | No reduction in CRR on day 2 | (Heller and Cryer 1991) |
| 2 hypos-30mins (2.8mmol/L) mane and (3.0mmol/L) afternoon on day 1 | Significant reduction in CRR on day 2 | (Heller and Cryer 1991) |
| 3 hypos- Less than 30mins – (<2.8mmol/L) on day 1, 2, 3 | No blunting of CRR on day 4 | (Peters, Rohloff et al. 1995) |
| Two hypos on day 1/or one hypo on day 1 and one hypo on day 3 and day 8 | If 2 hypos on day 1; Blunting of adrenaline/sweating on day 3 and 8 | (George, Harris et al. 1995) |
| | No blunting of noradrenaline or symptoms on day 8 | |
| Three (2 hour) hypos over 30 hours- protocol 1 /two (2 hour) hypos over 8 hours- protocol 2 | Blunting of CRR on day 5 only in protocol 1. | (Moheet, Kumar et al. 2014) |

3. Reduction in symptoms; this adrenergic response is closely linked with the symptomatic response to hypoglycaemia as both are driven by autonomic activation, and therefore when a reduced autonomic response is present, there is usually a reduced awareness of hypoglycaemia with the resultant increase in the risk of severe hypoglycaemia(White, Skor et al. 1983).

4. Defects in cortisol and growth hormone may additionally be responsible for causing longer duration hypoglycaemia with longer recovery times, with reduced cortisol causing a reduction in hepatic glucose production. Acute hypoglycaemia (AH) activates various pathways involved in the regulation of the neuroendocrine stress response. It is well established that prolonged hypoglycaemia activates the hypothalamic pituitary adrenocortical axis (HPA). However, a reduction in cortisol secretion is seen with RH, and has also been shown to contribute to a poor CRR (Davis, Shavers et al. 1996). Glucocorticoid deficiency with its resultant increase in whole body insulin sensitivity, has been shown in small case series to be associated with recurrent severe hypoglycaemia(Hardy, Burge et al. 1994).

Figure 2; The mechanisms behind the diminished counter-regulation and hypoglycaemia unawareness

Taken from (Cryer 2002).



As a result of the reduced glucagon, adrenaline and cortisol responses (defective glucose counter regulation) and the reduced symptom profile (hypoglycaemia awareness) see figure above, there is much morbidity in these patients with a 25 fold increase in the incidence of severe hypoglycaemia. Following is a review of the mechanisms proposed to date of the aetiology of defective counter regulation.

Mechanisms of counter-regulatory failure

Recurrent hypoglycaemia has been shown in multiple studies to be primarily responsible for the impairment of the counter-regulatory responses to further hypoglycaemia in both healthy individuals (Heller and Cryer 1991) and in those with T1D (Dagogo-Jack, Craft et al. 1993)

(see table 3). Recurrent hypoglycaemia appears to cause a reduction in the magnitude and threshold for stimulation of CRR responses. Several mechanisms as well as several brain regions are thought to be involved in the development to impaired CRR..

For instance, in a study involving rodent models using cFos activation as a label of cellular activity, it was shown that following RH, there is a diminishing response not only in the hypothalamus but also in other areas of the brain such as the paraventricular hypothalamus (PVH), dorsal medial hypothalamus (DMH), lateral hypothalamus area (LHA), paraventricular nucleus of the thalamus (PVT), nucleus tractus solitarius (NTS) and area postrema. This attenuation seems to be dependent on both extent and duration of the hypoglycaemia.(Paranjape and Briski 2005). This study and others demonstrate that many brain regions may directly, or indirectly be involved in the detection of hypoglycaemia.

Others have explored the way the brain senses and responds to low glucose. The brain contains populations of specialised neurons that respond directly to a change in the glucose level to which they are exposed with a change in membrane potential and firing rate. These glucose response (GR) neurons are divided into glucose-excited (GE) neurons that are activated when glucose levels rise and glucose inhibited (GI) neurons that are activated when glucose levels fall. In rodent models, the ability of GR neurons (both GE and GI neurons) to sense hypoglycaemia is dysregulated following recurrent hypoglycaemia. GE neurons exhibit a decreased response to low glucose as demonstrated by a lower threshold (0.1 instead of 0.5mM) before GE neurons are inhibited(de Vries, Arseneau et al. 2003). Similarly, GI neurons are also less able to sense decreased glucose after RH(Song and Routh 2006).

The mechanism for the change in glucose responsivity of GR neurons following RH is unknown but may reflect a change in the metabolism of glucose within the cell. Several investigators have suggested a key role for glucokinase as a regulator of neuronal

glucosensing (Dunn-Meynell, Routh et al. 2002). Inhibition of glucokinase has been shown to reduce activity in glucose excited (GE) neurons and increase glucose inhibited (GI) neuronal activity (Dunn-Meynell, Routh et al. 2002). This pattern of GE/GI change has been shown to increase the counter-regulatory responses (Routh 2002). Conversely, up regulation of glucokinase mRNA has been linked with the development of defective CRR, through potentially increasing the metabolism of glucose and therefore generation of ATP. This would lead to a lowering of the glucose level where glucose responsive (GR) neurons are activated (Kang, Dunn-Meynell et al. 2006).

Nitric oxide (NO) has also been shown to play a part in glucose sensing particularly in the GI neurons. AMP-activated protein kinase (AMPK) activation during periods of low glucose in GI Neurons leads to phosphorylation of the neuronal nitric oxide synthetase (nNOS) and production of NO which through binding to soluble guanylyl cyclase (sGC), increases cGMP levels. Adequate cGMP levels is essential for AMPK activation, chloride channel activation and consequently results in increased GI neuronal activation (Murphy, Fakira et al. 2009). However in models of RH (insulin injected for 3 consecutive days) the increase in NOS is severely blunted (Fioramonti, Marsollier et al. 2010) and this can theoretically affect GI neuronal activity and decrease the counter-regulatory response. It is also known that insulin induced hypoglycaemia down regulates mitochondrial free radical scavenger systems and increases reactive oxygen species (ROS) (Singh, Jain et al. 2004). The combination of increased NOS production in the presence of ROS, has been shown to increase S-nitrosylation of key proteins such as nNOS, which in turn decreases their activity, and subsequently may adversely affect GI neuronal glucose sensing (Jaffrey, Erdjument-Bromage et al. 2001).

Alternatively or in parallel, RH may impair the release of neurotransmitters from key GR neurons resulting in suppression of the neural networks involved in the detection of

hypoglycaemia and initiation of a CRR response. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. Recurrent hypoglycaemia has been shown to result in a significant increase in GABA levels (3 times higher) in a key brain glucose sensing region, the ventromedial hypothalamus (VMH) associated with an increase in GAD₆₅ (a protein that is associated with GABA turnover), resulting in a higher inhibitory tone (Chan, Cheng et al. 2008).. Increased GABA inhibitory tone would be predicted to lead to decreased action potential (AP) frequency in glucose sensing neurons, with suppression of the CRR during subsequent hypoglycaemia(Chan, Cheng et al. 2008).

Another modulator of the CRR system is the hypothalamo-pituitary-adrenal (HPA) axis. It has been suggested that modulation of the CRR, is through preferential activation of one of its receptors. VMH microinjection of CRH, ACTH and corticosterone, which acts primarily through CRHr1 amplifies the counter regulatory response (McCrimmon, Song et al. 2006). In contrast, urocortin-3, a member of the CRH family of neuropeptides, shows a suppressive action on the counterregulatory response through activation of the CRFr2 (CRF receptor 2) (McCrimmon, Song et al. 2006).

Recently, key ion channels within the brain have been identified in modulation of the CRR, notably the K_{ATP} channels as well as the glucose transporters. Studies with rodent models (Kir 6.2-) lacking both K_{ATP} channels and VMH GE neurons show a blunted glucagon response and significant defective counter-regulation, suggesting an important role of GE neurons (which contain K_{ATP} channels) in counter-regulation(Miki, Liss et al. 2001).

Moreover agents that modulate the K_{ATP} channels such as sulphonyureas (a K_{ATP} channel closer) (Evans, McCrimmon et al. 2004) and diazoxide (K_{ATP} channel opener) (McCrimmon, Evans et al. 2005), have been shown to modulate the CRR in rodent models. For example,

oral diazoxide has been shown to augment the counterregulatory responses during acute hypoglycaemia in rodent models. One potential explanation for this, suggests that when diazoxide opens the channel in the GE neurons, there is inactivation of the neurons. This consequently lifts the tonic inhibition placed on the GI neurons (usually from the activated GE neuron), which then stimulates the counter-regulatory response. However, as K_{ATP} channels are found throughout the brain, including other non-glucose sensing neurons, its promiscuity can cause many unwanted side effects.

An increase in the transport of glucose (Koranyi, Bourey et al. 1991) and alternative fuels into the glucose sensing neurons has been shown in animal models exposed to RH. However, recent PET studies have shown that after intravenous injection of ($1^{-(11)}C$ glucose), there was no change in glucose transport between those with T1D who had antecedent hypoglycaemia and those who were exposed to chronic hyperglycaemia. (Fanelli, Dence et al. 1998) In contrast other imaging studies have shown reduced glucose uptake and activity in the hypothalamus (Cranston, Reed et al. 2001), the prefrontal cortex (Bingham, Dunn et al. 2005), the amygdala and the orbitofrontal cortex (Dunn, Cranston et al. 2007) but with greater synaptic activity particularly in the dorsal midline thalamus (DMT) (Arbelaez, Powers et al. 2008) in those with T1D and IAH.

Extra metabolic substrates may also be obtained from local sources such as brain glycogen. Rodent models (Herzog, Chan et al. 2008) have shown that although there is an increase (super compensation) immediately after restoration of euglycaemia, levels are restored to normal within a few hours (a time when there is still defective counter-regulation), so glycogen super compensation is unlikely to be primarily responsible for the defective counter-regulation. This was confirmed in a recent study using in vivo (^{13}C nuclear magnetic resonance spectroscopy in association with radiolabelled glucose showed an overall lower glycogen content in patients with (T1D) with IAH than in controls. (Oz, Tesfaye et al.

2011) Lactate, a metabolite that can be taken up into neurons via monocarboxylate transporters however has been shown to be increased by 5 fold during a hypoglycaemic clamp. (De Feyter, Mason et al. 2013). This adaptation may mean that the metabolic needs are met, such that subsequent hypoglycaemia does not elicit an appropriate response with suppression of both CRR and decreased symptom response.

Peripheral components of the sympathoadrenal system (SAS) have also recently appeared to be affected as demonstrated in a recent rodent model. Animals exposed to RH showed a subsequent intact response to acetylcholine, with a much blunted adrenaline response suggested that this was a stimulus specific defect. This was thought to be as a result of a reduced efficacy of splanchnic nerve stimulation on the adrenal medullary chromaffin cells (Orban, Routh et al. 2014). Opioid generation during hypoglycaemia has been proposed in the generation of this defective CRR. A study showed that when opioid receptors were blocked with Naloxone, the sympatho-adrenal responses to hypoglycaemia was preserved. (Caprio, Gerety et al. 1991)

Although presence of autonomic neuropathy (AN) can contribute to the diminished symptom complex in IAH, it is not thought that presence of AN can solely contribute to IAH. This is confirmed when studies show that complete exclusion of hypoglycaemia over a period of time, can restore symptoms associated with hypoglycaemia even in the presence of AN. Also, there appears to be no relationship between autonomic dysfunction and hypoglycaemic symptoms (Berlin, Grimaldi et al. 1987).

In summary, counter-regulatory failure has been studied through examination of various cellular models. Mechanisms proposed have included increases in GABA, upregulation of glucokinase mRNA, blunting of the NOS, increases in ROS, as well as an increase in the metabolism of glucose and/or alternate fuels. In addition, various modulators of the CRR

have also been studied and include the CRH family of neuropeptides, and the opioids. Animal models have suggested changes in the glucose uptake in the brain, but this is yet to be replicated in humans. Alternate fuels such as lactate have shown to be a valuable source of energy when glucose is deficient, maintaining the cell's metabolic needs and appears to be a leading hypothesis for adaptation in this context.

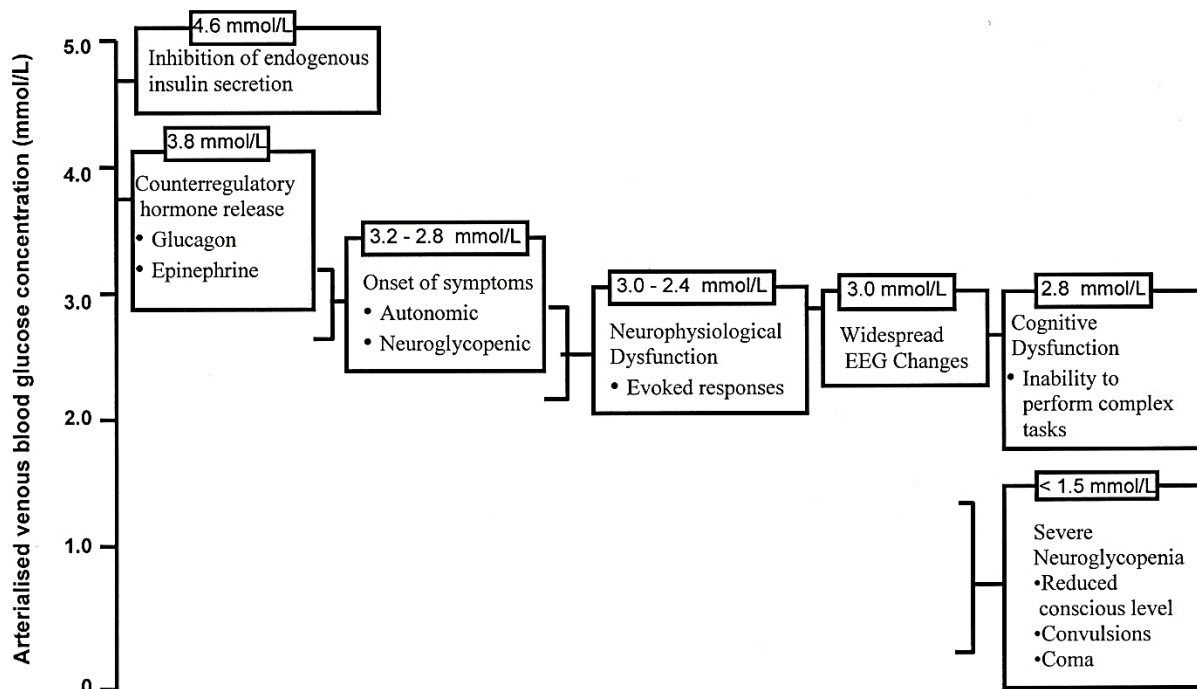
Thresholds

Not only is the magnitude of the counter-regulatory responses (CRR) significantly diminished but the glucose thresholds at which the various components of this response are initiated are also altered in those experiencing recurrent hypoglycaemia. Therapies therefore need to focus not only on increasing the magnitude of CRR, but should also strive to improve thresholds in order for patients to be given adequate time and warning, before experiencing a severe hypoglycaemic episode.

Generally in a non-diabetic individual thresholds are fairly static and counter-regulatory hormones are activated at a plasma glucose threshold of ≈ 3.6 - 3.8 mmol/L, autonomic symptoms appear at ≈ 3.2 mmol/L, with neuroglycopenic symptoms at approximately 2.8mmol/L and cognitive function deteriorates at about 2.8 mmol/L(Mitrakou, Ryan et al. 1991). This hierarchy of responses ensures that blood glucose is kept at a very tight range in a non-diabetic individual. Also, this hierarchy gives the patient ample time before cognitive function is affected.

Figure 3; Glucose thresholds for activation of CRR hormone release, autonomic and neuroglycopenic symptoms and cognitive deterioration in the non-diabetic human.

(Frier 1999)



Several methods have been used to calculate thresholds. The most reliable of which is the hyperinsulinemic hypoglycaemic clamp technique.

Methods used have been;

- Plasma glucose at which mean responses were significantly different from the mean respective values in a time-matched euglycaemic study
- Plasma glucose at which the mean response exceeds the 95% CI for that parameter at the corresponding time during the euglycaemic arm. (Schwartz, Clutter et al. 1987, Mitrakou, Ryan et al. 1991)
- Plasma glucose at which mean response exceeds a predetermined increment from the mean values calculated during the euglycaemic phase of the trial. (Adrenaline-75pg/ml; noradrenaline-50pg/ml; growth hormone-7mg/ml; and cortisol 7mcg/dL).

The adrenaline increment represents a clinically meaningful rise. The other

increments were based on average responses of these hormones during an insulin tolerance test of pituitary adrenal axis function. This method did not require a euglycaemic arm(Amiel, Sherwin et al. 1988).

- d. Plasma glucose at which mean response is greater than 2 SD or a doubling of the euglycaemic values.

However these thresholds are far more dynamic in an individual with diabetes and can be influenced by a number of factors such as prevailing glucose levels and level of metabolic control. It has been shown that even a short period of intensive insulin therapy (3-6 months) in newly diagnosed patients with T1D, results in a consistent increase of the glucose threshold (2.6mmol/L) for adrenaline release with markedly lower adrenaline concentrations ultimately leading to a diminished symptom complex(Hoffman, Arslanian et al. 1994). It was also noted that the thresholds for the other counter-regulatory hormones were also increased. (Amiel, Sherwin et al. 1988). The converse is true in those with poorly controlled diabetes (lower glucose threshold) compared to non-diabetic subjects. (Boyle, Schwartz et al. 1988). The thresholds however are not dependent on the rate of fall, with rates up to 9 fold difference in plasma glucose level not affecting glucose thresholds for CRR hormone release(Amiel, Simonson et al. 1987).

Impact of defective counter regulation

Impaired Awareness of Hypoglycaemia

It is imperative that an individual has adequate symptoms to alert them of impending hypoglycaemia, to allow them to self-treat and prevent severe hypoglycaemia. It has been shown that both autonomic and neuroglycopenic symptoms contribute equally to the symptomology alerting an individual to impending hypoglycaemia (Cox, Gonder-Frederick et al. 1993). However, RH has been shown to increase the glucose thresholds at which

symptoms and counter-regulatory response are generated (Heller and Cryer 1991, Dagogo-Jack, Craft et al. 1993, Davis, Mann et al. 2000). The combination of a defective counter-regulatory response and a diminished symptom complex during hypoglycaemia has been termed Impaired Awareness of Hypoglycaemia (IAH). In patients with IAH, it is thought that there is a significant delay for autonomic activation, and hence relatively little sensory feedback to alert the patient of falling blood sugars. This then quickly leads to neuroglycopenia (confusion, drowsiness, odd behaviour, speech difficulty, and inco-ordination). IAH is associated with a six fold increase in severe hypoglycaemia and is a cause of major morbidity (Gold, MacLeod et al. 1994).

Several hypothesis are currently being tested as to why patients develop IAH. One of the popular viewpoints is illustrated below (Figure 4). Hypoglycaemia is a significant stress to the cell and leads to energy depletion. This normally would lead to sympathetic activation, and in severe cases lead to cell death. However with RH, the cell adapts by using alternate fuels and alters its glucose metabolism such that during subsequent hypoglycaemia, there is less energy depletion. This can be seen as an adaptive process with less cell death, but is also maladaptive with a significant diminishing of the sympathetic response with reduced symptom generation (hypoglycaemia unawareness) and a reduced adrenomedullary response and ultimately an increased risk of severe hypoglycaemia.

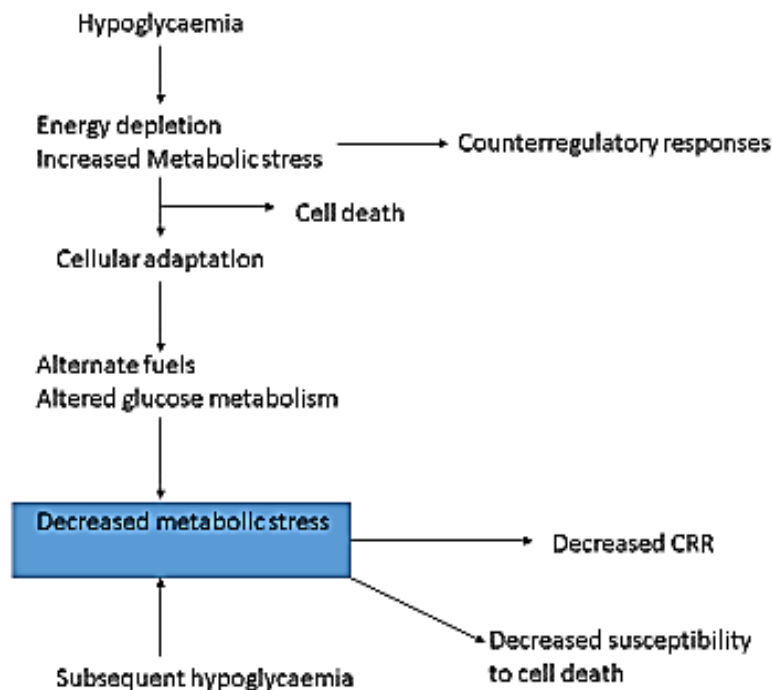


Figure 4. Proposed model for development of hypoglycaemia associated autonomic dysfunction (HAAF)

Adapted from (Martin-Timon and Del Canizo-Gomez 2015). Acute hypoglycaemia can be seen as a stressor with appropriate physiological responses (Counterregulatory responses), however severe hypoglycaemia can lead to cell death, but with recurrent hypoglycaemia, there is less metabolic stress with reduced responses, which may be protective with less cell death.

There is still much debate about how to define IAH, with the proposed methods listed below;

- a. A diminished ability to detect the onset of acute hypoglycaemia.
- b. Gold score ≥ 4 or Clark score ≥ 4
- c. Failure to develop symptoms during experimental induction of hypoglycaemia.
- d. Failure to mount an adequate CRR hormonal response to hypoglycaemia. ($>2SD$ from mean basal levels)

Use of questionnaires have enabled researchers to determine the extent of the problem in patients with T1D. In the UK Hypoglycaemia Study, the incidence of IAH was 7% in those with short duration T1D, and 35% in those with duration of disease over 15 years(Choudhary, Geddes et al. 2010)A recent cross sectional study in 445 patients with T1D showed that a

longer duration of diabetes was associated with lower intensity of autonomic symptoms and higher prevalence of impaired awareness (with 3% for duration 2-9 years to 28% for duration ≥ 30 years).

Effect on cognitive ability

Effects during acute hypoglycaemia (in those with RH)

It appears that those who have IAH perform better on cognitive tests during hypoglycaemia than those who have normal awareness. In this group, it has also been found that those with IAH, have a quicker recovery time of cognitive ability once euglycaemia is restored. (Zammitt, Warren et al. 2008, Stalder, Evans et al. 2010) This suggests that IAH can be viewed as both an adaptive and maladaptive condition.

Long term effects of recurrent hypoglycaemia on cognitive function

The DCCT and its 18 year follow up study have shown recently, that when cognitive tests were done regularly, that those with SH showed no deterioration of these neurocognitive tests as opposed to those without SH. (Diabetes, Complications Trial/Epidemiology of Diabetes et al. 2007) However using newer imaging techniques, it appears that greater volumes of the brain are recruited to maintain the cognitive function during hypoglycaemia. (Bolo, Musen et al. 2011), however this may be at the risk of poor brain efficiency.

However,, a recent review showed that those with T1D had impaired cognition with an effect size of 0.3-0.8 SD units compared to non-diabetic controls. (McCrimmon, Ryan et al. 2012)

The main cognitive domains that were affected were of information processing speed, mental flexibility and psychomotor functioning. It was concluded that the main risk factor for developing these complications was the presence of microvascular complications rather than occurrence of SH or poor glycaemic control. (Brands, Biessels et al. 2005) Numerous studies

have consistently found that T1D results in a decrease in hippocampal cell proliferation and survival (Balu and Lucki 2009), which may also be related to HPA axis dysregulation (Revsin and de Kloet 2009).

One of the factors which may provoke cognitive decline, is the experience of severe hypoglycaemia (SH) before the age of 5, which resulted in problems with spatial intelligence and delayed recall later on in life (Perantie, Lim et al. 2008). However a study in older adults (60.4 \pm 4.6 years) showed that those who had SH were more susceptible to cognitive decline particularly in information processing speed, which suggested that in the older adult, the brain is more vulnerable to the effects of hypoglycaemia. (Duinkerken, Brands et al. 2011)

Therefore it appears that hypoglycaemia per se, does not cause any long term cognitive decline and rather it is presence of microvascular complications that may confer a greater risk in development of cognitive problems. However the very young and elderly tend to be more vulnerable in sustaining chronic injury. The potential impact on cognition, may also account for a reduced adherence to suggested changes to medications in patients with long duration diabetes. (Smith, Choudhary et al. 2009, Marketou, Kalyva et al. 2014).

Effect on the Hypothalamo-pituitary axis (HPA)

Hypoglycaemia can be considered to be a stressor and activates the paraventricular nucleus of the hypothalamus, which then secretes corticotropin releasing hormone (CRH). This molecule is carried through the hypophyseal portal circulation to the anterior pituitary gland, which consequently secretes adrenocorticotropin hormone (ACTH). ACTH carried through the peripheral circulation stimulates the adrenal gland to make and release cortisol in the zona fasciculata.

Recurrent hypoglycaemia on the other hand is thought to lead to a reduction in cortisol response similar to the counter-regulatory hormones, but to a lesser extent (Mokan, Mitrakou et al. 1994). Antecedent glucocorticoids have been shown to reduce the magnitude of catecholamine release during next day hypoglycaemia (Davis, Shavers et al. 1996) Similarly pre-treatment with multiple treatments with CRH but not ACTH or cortisol was found to suppress the next day hypoglycaemic CRR(Flanagan, Keshavarz et al. 2003). This effect is thought to be mediated by the CRH receptors with CRH-R2 mediated effects leading to suppression and CRH-R1 amplifying the CRR to hypoglycaemia(Bale and Vale 2004). Interestingly, glucocorticoids either endogenous or exogenous can lead to an increase in the CRH R2; R1 ratio. This rise in endogenous glucocorticoids (as determined by the depth and duration of hypoglycaemia) during AH may explain why there is a reduction in catecholamine release during a subsequent hypoglycaemia (Davis, Mann et al. 2000).

There is currently very little in the literature on the effects of recurrent hypoglycaemia on the diurnal variation of the HPA axis in humans and therefore the literature on the impact of other recurrent stressors on the HPA system may be illuminating. Certainly exposure to a chronic stressor is known to increase the vulnerability to poor health outcomes. (Cohen, Frank et al. 1998)

Thus recurrent hypoglycaemia (RH) has widespread deleterious impact on several systems, with diminishing sympathoadrenal activation and symptom generation and possible altered cognition (particularly in the extremes of life-very young and very old). RH may also through recurrent activation of the stress systems-HPA axis lead to more widespread maladaptive changes through alteration of the diurnal patterns of cortisol release and is the focus of STUDIES 3 and 4.

CHAPTER 2

Therapeutic strategies employed to improve Impaired Awareness Hypoglycaemia

Problems with current Insulin preparations

Despite newer insulin analogues and new insulin delivery devices, the overall rates of severe hypoglycaemia in epidemiological studies have remained unchanged over the last 20 years (Bulsara, Holman et al. 2004). Although more recently, Bulsara et al, reported that in the Western Australia children's database (age range 1-18 years), rates of severe hypoglycaemia had fallen over the last decade with the nadir in 2006 (17.3 to 5.8 events per 100 patient years)(O'Connell, Cooper et al. 2011).

Despite the improvement in the pharmacodynamic properties of newer insulins, exogenous insulin still requires to be delivered subcutaneously rather than directly into the portal vein. In normal physiology 100% of endogenous insulin (released every 4-5mins throughout the day) flows from the pancreas to the liver(Geho 2014). This insulin, during fasting, directly inhibits hepatic glucose release by inhibiting gluconeogenesis, glycogenolysis and also indirectly through the suppression of α -cells, glucagon release. The latter occurs because blood flow in the islet is such that α -cells are exposed to high intra-islet levels of insulin and other factors released by the β -cell which are tonically inhibited by insulin. However, post-prandial endogenous insulin stimulates glucose storage by the liver. The remaining insulin released by the liver, also acts on the two other insulin sensitive tissues (adipose and skeletal muscle) and stimulates glucose uptake. Up to 80% of endogenously secreted insulin is degraded on its first pass through the liver (Triplitt and Chiquette 2006). This means that in non-diabetic individuals there is a significant portal-peripheral insulin gradient with a 2-3-

fold higher insulin concentration in the portal vein as compared to that seen in the systemic circulation.

In type 1 diabetes this physiological state can only be achieved if there is a high systemic level of insulin to ensure portal insulin levels are adequate for suppression of α -cell glucagon secretion. This results in systemic hyper-insulinaemia which may have adverse cardiovascular consequences and has recently been implicated in the development of insulin resistance (Okamoto, Anhe et al. 2011). Moreover, a depot of exogenous insulin is ‘unregulated’, i.e. not influenced by the many alternate signals that modify pancreatic β -cell insulin secretion. This is most obvious during hypoglycaemia where a failure of insulin to ‘switch-off’ contributes both to the progressing hypoglycaemia and to a specific defect in the ability of subjects with type 1 diabetes to secrete glucagon in response to low glucose (Ringholm, Pedersen-Bjergaard et al. 2012).

Moreover, if there are insufficient levels of portal insulin, such that there is a reduced ability of the liver to store ingested carbohydrates as glycogen, this can further predispose patients to potential problems. Consequently peripheral tissues have to dispose most of the carbohydrates and therefore require even more insulin to regulate post-prandial hyperglycaemia. Secondly, the inadequacy of liver glycogen storage predisposes the patient to more severe hypoglycaemia.

Furthermore, studies have shown a cohort of patients who are reluctant to change their medication regimen despite recurrent hypoglycaemia. A PET study has shown that there is a pattern of decreased activation of stress pathways during hypoglycaemia in subjects with T1D and IAH with a preferential preservation of pathways which are involved with motivation and reward (Dunn, Cranston et al. 2007), which may be as a result of repeated exposure to opioids (β endorphins). This may influence patients’ perception of

hypoglycaemia with possible pleasurable and addictive phenomena associated with recurrent hypoglycaemia or conversely that a sense of “threat” to well-being is not perceived.

In summary, the non-physiological delivery of exogenous insulin, the almost inevitable burden of hypoglycaemia and the problems associated with recurrent hypoglycaemia such as IAH and the addictive phenomena all suggest that there needs to be further study in either improving the mode of delivery of insulin or adding in adjunct therapies to counter the above problems.

Current treatment strategies

As is now well established, recurrent hypoglycaemia leads to a suppression of the symptom and hormonal complex. Meticulous avoidance of biochemical hypoglycaemia for 2 weeks (Fanelli, Epifano et al. 1993) has been shown to improve the autonomic symptoms (with complete restoration occurring after 3 months) and results in an alteration of glycaemic thresholds for adrenaline release, symptoms and cognitive function. However studies which have concentrated on meticulous avoidance of hypoglycaemia have been highly labour intensive, requiring considerable input from the study team (Fanelli, Epifano et al. 1993, Cranston, Lomas et al. 1994), a model which would be difficult to replicate in real life clinical settings. Several alternative strategies such as taking snacks in between meals and at bedtime (Cranston, Lomas et al. 1994) and raising blood sugar targets (Fritzsche, Stefan et al. 2001) have been employed in an attempt to achieve complete avoidance of BH but are often associated with worsening glycaemic control.

Studies have shown that improvement of the deficient glucagon and adrenaline responses does not necessarily mean improvement of the symptom generation, implying there are different mechanisms underlying symptoms and the generation of the neuroendocrine responses. (Dagogo-Jack, Rattarasarn et al. 1994)

A number of non-drug strategies have been trialled to enable patients to try and reduce the incidence of recurrent hypoglycaemia and IAH with varying effects.

Non-drug strategies for reducing hypoglycaemia

Education

Detection of hypoglycaemia

As the magnitude of catecholamine release diminishes with time and with recurrent exposure to hypoglycaemia, the symptom complex diminishes(Graveling and Frier 2010). Typical autonomic symptoms and even neuroglycopenic symptoms may not occur and patients are then increasingly reliant on atypical symptoms (strange sensation around mouth, “funny feeling” etc.), and in the worst case scenario, patients have no awareness of hypoglycaemia and rely on those around them to prompt them. Patients and their families need to be taught about recognizing the symptoms of hypoglycaemia and to be familiar with appropriate treatment with an emphasis on not delaying treatment. Blood Glucose Awareness Training (BGAT) programs has also been shown to be an effective way for both patients and partners to pick up subtle cues of evolving hypoglycaemia and then to respond promptly to prevent more SH. It was initially designed as 8 weekly group sessions, and showed both improvement in awareness and reduction in SH at 6 and 12 months(Cox, Gonder-Frederick et al. 1995, Fritsche, Stefan et al. 2001).

Education programmes

When therapies are used to intensify treatment, one would expect rates of hypoglycaemia to increase but education programmes running alongside such therapy have been shown to attenuate this risk.(Samann, Muhlhauser et al. 2005) In contrast to the increase in severe hypoglycaemia seen in the intensive arm of the DCCT cohort, the UK DAFNE(Dose adjustment for Normal Eating) have shown that the improvement in glucose control can be

achieved with a decrease (1.7 ± 8.5 to 0.6 ± 3.7 episodes per person per year) in severe hypoglycaemia. The DAFNE cohort (40% IAH at entry) showed that 43% of patients with hypoglycaemia unawareness, were able to improve recognition, after 1 year of training. (Hopkins, Lawrence et al. 2012) Due to this resistance to change behaviour in certain groups, DAFNE-HART was designed to employ motivational interviewing and cognitive behavioural techniques in those who had attended the DAFNE insulin educational program, but still had persistent IAH. This program showed after 12 months following intervention, that rates of SH fell from 3 to 0 ($P < 0.0001$) per person per year and moderate hypoglycaemia fell from 14 to 0 ($p < 0.001$) per person per 6 weeks. Perceptions around hyperglycaemia also improved (de Zoysa, Rogers et al. 2014)

The recent HypoCOMPASS trial (Leelarathna, Little et al. 2013) which utilised a 2 by 2 factorial design was designed to compare the impact of real time –continuous glucose monitoring (RT-CGM) and continuous subcutaneous insulin infusion (CSII) vs Multiple Daily Injections (MDI) on hypoglycaemic awareness. The educational tool was given to all participants prior to entry onto the trial, it taught patients to recognise the subtle symptoms associated with hypoglycaemia, never to delay the treatment and to recognise periods of increased risk, particularly at night. At the end of the 24 week trial, there was similar glycaemic outcomes with both conventional MDI and SMBG regimens compared with CSII/RT. It has been proposed that the frequent contact with the research team may have explained any lack of difference, with possible contamination between the 4 groups.

Patients often get into set routines in order to combat hypoglycaemia, with some accepting hypoglycaemia as part and parcel of their condition and therefore failing to recognise avoidable precipitating factors or delaying treatment, with the experience of recurrent hypoglycaemia then leading to significant morbidity particularly with the development of IAH. Therefore education programs seek to re-train an individual in not only quick

identification of subtle cues of hypoglycaemia but also to identify preventable precipitants, and then to engage in quick, effective treatment. Furthermore, the activation of cerebral networks involved with reward associated with hypoglycaemia, may further impinge the ability of certain individuals to change behaviour.

Technology

Continuous Glucose Monitoring (CGM) Systems

The US Food and Drug Administration (FDA) advocate using CGM in the following situations: detecting trends of glucose values, an adjunct to self-monitoring of blood glucose (SMBG), aiding in the detection of hyperglycaemia or hypoglycaemia, minimising glucose excursions and facilitating both acute and long term therapy adjustments.

CGM operates by measuring the glucose levels in the interstitium. An applicator is used to insert the 21-26 gauge devices into the interstitium. The Medtronic systems have a disposable sensor that generates a current as a result of the ambient glucose and provide measurements every 5 minutes. This signal is sent to the transmitter. The sensor values are paired with calibration blood glucose readings in a retrospective manner, to ensure accuracy. The latest versions have a mean absolute relative difference (MARD) of <15% suggesting reasonable accuracy.

RT-CGM is able to display the blood glucose, but more importantly the direction and the rate of change and therefore gives the wearer more information than a single reading in order to proactively avoid hypoglycaemia. CGM has the advantage of a semi-continuous flow of glucose readings, which may enable insulin dose decisions.

The use of CGM has shown to improve HbA1c without increasing the incidence of hypoglycaemia (Deiss, Bolinder et al. 2006, Juvenile Diabetes Research Foundation

Continuous Glucose Monitoring Study, Tamborlane et al. 2008, Beck, Hirsch et al. 2009) with greatest effect in those with a higher HbA1c and with increased usage of sensor (Pickup, Freeman et al. 2011). However, in contrast, two studies (Pepper, Steinsapir et al. 2012, Duran-Valdez, Burge et al. 2014) investigated the utilisation of blinded CGM/professional monitoring systems. Firstly, it was shown that the magnitude of correlation between average interstitial glucose as determined by the CGM and A1c was too low ($R^2 < 20\%$) with limited ability to improve HbA1c. These studies show that the blinded CGM may have limitations for improving A1c target, but may be more appropriate for identifying periods of nocturnal hypoglycaemia and other periods of glucose fluctuation (a measure that is not identified by HbA1c).

The JDRF-CGM study did not however, show a reduction in hypoglycaemia. Reasons for this included that patients were found to sleep through 74% of alarms (Buckingham, Block et al. 2005) and because of sensor inaccuracies (Leelarathna, Nodale et al. 2013). However, the JDRF-CGM study showed that CGM readings with 30% or more of the readings in the hypoglycaemic range were associated with an 8 fold increase in SH the following day, however because SH was a relatively uncommon event, this was shown to have a poor positive predictive value (PPV) ($< 5\%$). (Fiallo-Scharer, Cheng et al. 2011). Two possibilities were given for this poor PPV: Firstly, that of the patient altering behaviour after the hypoglycaemic episode. Secondly, the measurement error from CGM. The median error has been quoted during hypoglycaemia to range between 0.7 to 1.3 mmol/L, which means there can be both over and underestimation with CGM devices. (Wilson, Beck et al. 2007, 2008) In the univariate analysis of this study, further risk factors for SH were ascertained; higher scores on the Hypoglycaemia Fear Survey ($p=0.02$), those with higher percentage of CGM values below 3.9 mmol/L and those with a higher GV measured with coefficient of variation ($p=0.08$). However all these factors could be as a result of previous SH. In the multivariate

analysis, only SH and female sex were found to be independent predictors of SH. A further study looking at rates of SH over 6 months before and after CGM, showed rates of SH reduced from 21.8 to 7.1 events per 100 patient years with on average patient wear of 6.8days/week. (Bode, Beck et al. 2009)

However, a paediatric prospective study (Ly, Hewitt et al. 2011) recently showed that wearing RT-CGM with the use of low glucose suspend alarms set at 6mmol/L for a 4 week period was able to significantly improve the adrenaline response at 2.8mmol/L during a hyperinsulinemic hypoglycaemic clamp. ($1,093 \pm 221$ vs 572 ± 162 pmol/L $p=0.048$). The recent ASPIRE trial has shown that using the automated suspension when BG reaches 3.9mmol/L, the duration (19% reduction) and severity of hypoglycaemia was significantly less ($p=0.006$) without significant rebound hyperglycaemia (Garg, Brazg et al. 2012) however a further analysis showed that the ability to mitigate this hypoglycaemia was dependant on the order of the studies, with more significant reduction in hypoglycaemia if the feature was used first. (Garg, Brazg et al. 2014) A recent further study evaluating the effectiveness of alarms in those using CGM showed that irrespective of alarms, those wearing CGM had significantly lower time spent outside the glucose targets compared to SMBG. This effect was greater when CGM was associated with CSII therapy. Presence of the alarms however did not deter patients from wearing the system (New, Ajjan et al. 2015). However there is a reported 30% false alarm rate (Kamath, Mahalingam et al. 2010), which if perceived by the patient, can affect the response rates. It may therefore be pertinent to include alarm coping strategies during education (Shivers, Mackowiak et al. 2013).

The major disadvantages of CGM technology is the time lag (which may be a couple of minutes), which may cause confusion regarding treatment decisions, particularly, when there is a discrepancy between CGM and peripheral SMBG readings. Moreover, the constant provision of information may in certain patients be off putting and be considered as a

permanent reminder of their illness. As each sensor needs to be replaced every 5-7 days, there is also a cost implication of long term use of CGM. In summary, real time CGM appears to have benefits in improving glycaemic control without worsening hypoglycaemia, with blinded CGM only being useful for identifying periods of high risk. Further advantages have been seen when they are used with the low glucose suspend feature in a closed loop system, and also with alarms, although alarm fatigue can pose a problem. However the major disadvantage is that of sensor inaccuracies particularly during hypoglycaemia, although this is improving with the newer generation CGM devices.

Continuous Subcutaneous Insulin Infusion (CSII)

A meta-analysis comparing CSII with MDI (Pickup and Sutton 2008) showed that there was a 0.3-0.6% improvement in HbA1c and a 4 fold reduction in severe hypoglycaemia, in those with the highest baseline HbA1c levels and highest rates of SH, with the group on MDI benefiting the most after conversion to CSII. A further review of 26 observational studies confirmed that CSII significantly decreases rates of severe hypoglycaemia. (Cummins, Royle et al. 2010) The SWITCH study (Battelino, Conget et al. 2012) looking at the addition of CGM to CSII with its crossover design eliminating the most frequently encountered confounders of diabetes related education, showed a significant reduction in HbA1c (difference - 0.41% (95% CI - 0.28%, - 0.53%; $p < 0.001$) with a reduction in time spent with glucose levels less than 63mg/dL (3.5mmol/L) (19 vs. 31 min/day, respectively; $p = 0.009$).

A prospective study in people with IAH, showed that transitioning patients onto CSII from multiple daily injections not only halved the frequency of hypoglycaemia but also reduced rates of SH from 1.25 to 0.05 events per year. (Gimenez, Lara et al. 2010)

The meta-analysis of RCTs by Yeh et al comparing MDI with CSII and the monitoring strategies (SMBG vs RT-CGM) failed to show any change in SH and although HbA1c was

improved with CSII, it was heavily influenced by one study (due to higher baseline HbA1c) (DeVries, Snoek et al. 2002, Dupre, Behme et al. 2004) with the effect on HbA1c nullifying when this study was removed.

More recently, the HypoCOMPASS study (Leelarathna, Little et al. 2013) compared the efficacy of optimised MDI versus CSII with and without sensors in a 2 by 2 factorial study design in patients with long standing T1D, who had all received the HypoCOMPASS educational tool prior to randomization. Despite an improvement in the concentration and threshold for secretion of plasma metanephrine during hypoglycaemia, the investigators failed to show an advantage of one treatment modality over the other. This was a landmark study, since prior to it, most of the studies compared CSII with MDI, where NPH was the comparator basal insulin, which has known problems of nocturnal hypoglycaemia. A further study (REPOSE-Relative Effectiveness of Pumps over MDI and Structured Education for Type 1 Diabetes) which is currently running, will also aim to understand the benefit of CSII over MDI on glycaemic control and hypoglycaemia when all subjects are receiving structured training in insulin therapy (DAFNE). (White, Waugh et al. 2014)

Although initial studies suggested that CSII over MDI had greater advantages of reducing SH and improving HbA1c, this was not found in a large meta-analysis by Yeh et al, with the HypoCOMPASS trial also not showing any superiority in either modality. However we await the reporting of the REPOSE trial before drawing any further conclusions.

Closed loop

The Sensor-Augmented Pump therapy for A1C reduction (STAR-3) trial showed that when combining CGM with CSII, there was a significant reduction in HbA1c (8.3 to 7.5%) compared to MDI with no CGM (8.3 to 8.1%). However it did not lead to a significant reduction in rates of SH (13.1 pump vs 13.48 injections persons per 100 person years). A

further study showed that if either MDI or CSII had access to CGM, then they were better able to manage extremes of glucose excursions. (Garg, Zisser et al. 2006)

More recently, Ly et al showed in the first randomized controlled trial that use of a sensor augmented insulin pump with the suspend feature (cf standard insulin pump therapy), in a group where all participants had IAH (mean Gold score of 6.4), resulted in a significant reduction in both severe (defined as seizure or coma) and moderate (defined as needing assistance) (175 to 35 episodes in the Low glucose suspend group) hypoglycaemia, as well as a reduction in sensor readings (using a blinded CGM) of lower than 60mg/dL and 70mg/dL (3.3 and 3.9mmol/L)(Ly, Nicholas et al. 2013). However in contrast to earlier studies, this study when using the low glucose suspend feature, did not show an improvement in adrenaline responses (to hypoglycaemia during a hyperinsulinemic hypoglycaemic clamp, (220pg/mL vs 148 pg/mL $p=0.26$) however there was an improvement in the Hypoglycaemia Unawareness Score (as determined by the Clark method) from 5.9 (95%CI 4.5-6.8) to 4.7 (95% CI 4.0-5.1) in the low glucose suspension group ($p<0.001$), once again comparable to earlier studies showing a dissociation between adrenaline responses and symptom scores(Ly, Nicholas et al. 2013).

In fact a large Cochrane collaboration review (22 studies with 2883 patients)(Langendam, Luijf et al. 2012) showed that the best outcomes were in those with poor glycaemic control who started using sensor augmented pump therapy, as opposed to MDI and SMBG, with HbA1c level falling by 0.7%, 95% confidence interval (CI) -0.8% to -0.5%, 2 RCTs, 562 patients, $I^2=84\%$). Those using CGM had a higher incidence of hypoglycaemia, however the confidence intervals was wide and included unity (95% CI 0.38 to 27.82). This analysis was confined to one study, with insufficient detail to analyse frequency of hypoglycaemia, however, the follow-up was short (6 months), and therefore conclusions cannot be drawn from this (with GRADE of evidence- very low)

Closed loop studies have shown variable results, but appear to be most beneficial in those with poor glycaemic control, and when the low glucose suspend feature is utilised. This technology is improving as sensor accuracy and algorithms are improving.

Transplantation

CGM has shown that those who have received transplantation either whole or islet cell significantly have reduced the time they spend in hypoglycaemia. This would be as a result of absence or reduction of exogenous insulin needed, a regulated insulin secretion pattern from the transplant and a partial restoration of both the glucagon and the sympatho-adrenal responses as a result of reduction in frequency of overall hypoglycaemia. (Paty, Senior et al. 2006). Recent records show that one year islet cell survival in the UK is 87%. Overall glycaemic control was improved from 66 ± 16 and frequency of SH was reduced from 23/patients per year to 0.56/patients per year. A recent ADA abstract (Hering et al, 2014) reported on the Clinical Islet Transplantation Consortium, which was a prospective, open label single arm study, whereby allogenic human pancreatic islet product was infused intraportally. All patients had reduced awareness of hypoglycaemia and at least one episode of SH. The median daily dose at 1 year post transplantation was 0 (0-0.43u/kg), with 94% having a functional graft. The median Clarke score reduced from a baseline 6 to 0 ($p < 0.001$) with a reduction in SH and indices of glucose variability ($p < 0.001$)

This currently is a strategy reserved for those with recurrent severe hypoglycaemia. This has become a relatively safe procedure, particularly since careful heparinisation (eliminating the previously feared complication of portal vein thrombosis) and the use of steroid free immunosuppression (based on sirolimus and tacrolimus) (Shapiro, Lakey et al. 2000).

In summary, patients with hypoglycaemia should have individualised therapy utilising the above models. A recent evidence informed clinical practice recommendation for those with problematic hypoglycaemia suggests hypoglycaemic specific education programs, with progression to technology, and then sensor augmented insulin pumps with transplantation reserved for those who are still having problems. (Choudhary, Rickels et al. 2015).

Drug strategies for reducing hypoglycaemia

Although the search for more physiological strategies for insulin replacement may address some of the problems, potential adjunct therapeutic options have also been presented in the literature to date, to either preserve or improve the counter-regulatory responses to hypoglycaemia by inducing a recovery of glucagon, modulating neurotransmitters and sensing mechanisms or through the use of dietary supplementations.

Approaches to address α cell defects

Role for amino acids

Within 5 years of disease duration, most patients with T1D have a blunted glucagon response to hypoglycaemia. Defects in the portal insulin to glucagon ratio, which is key in regulating hepatic glucose production, can lead to significant delays in glucose recovery following hypoglycaemia. Glucagon is probably the most important counter regulatory hormone and so restoration of this response would be beneficial in reducing severe hypoglycaemia risk.

Amino acids stimulate glucagon release from pancreatic α cells (Kuhara, Ikeda et al. 1991), however their ability to restore this response in type 1 diabetes is inconsistent, particularly when given intravenously (Caprio, Tamborlane et al. 1993, M'Bemba, Cynober et al. 2003, Porcellati, Pampanelli et al. 2007). In contrast, oral administration of amino acids enhanced glucagon responses ($p=0.016$) during hyper-insulinaemia hypoglycaemia in T1D subjects and

preserved some aspects of cognitive function (Rossetti, Porcellati et al. 2008). However, oral amino acids also stimulated glucagon release during euglycaemia and hence would be anticipated to have deleterious effects on overall glucose control.

Approaches to address central defects

Role of GABA

γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter, and is known to regulate multiple physiological compensatory processes. Rodent models have shown GABA levels to decrease during systemic hypoglycaemia (Beverly, De Vries et al. 2001). Pharmacological reduction of hypothalamic GABA has been shown to enhance the neuroendocrine responses to hypoglycaemia in rodent models.

Modafinil is an agent used in narcolepsy and reduces GABA activity probably through K^+ channels (Gulanski, De Feyter et al. 2013). Although the full mechanism of modafinil is still unknown, it may work by increasing dopamine in the brain by reducing the re-uptake of dopamine in the nerves. In a study of human subjects, modafinil resulted in a moderate increase in adrenergic symptoms and heart rate, but had no effect on the counter-regulatory responses to hypoglycaemia (Smith, Pernet et al. 2004). Nevertheless this effect could still be beneficial in improving subjective awareness of hypoglycaemia in those with IAH despite limited recovery of other counter-regulatory responses.

Role for Beta agonists

Noradrenaline, a monoamine neurotransmitter is released into the ventromedial hypothalamus (VMH), a key brain region involved in hypoglycaemia sensing and appears to regulate VMH function during hypoglycaemia.(Beverly, De Vries et al. 2001). Noradrenaline has been reported to stimulate glutamatergic neurotransmission and is thought to mediate its effect via the β_2 adrenergic receptors (B2AR). (Lee, Choi et al. 2007). Recent rodent studies have

shown that local VMH delivery of B2AR agonists increase both adrenaline and glucagon responses to hypoglycaemia. (Szepietowska, Zhu et al. 2011)

A recent study looking at the different methods of reducing the risk of nocturnal hypoglycaemia (Raju, Arbelaez et al. 2006), showed that night time administration of oral terbutaline (5mg), an oral β_2 agonist reduced the percentage of blood glucose values below 3.9mmol/l from 27% to 1% of the measured values however it did increase morning blood glucose values (37% higher) and increased heart rate and blood lactate levels. Dose defining studies may need to be done to study this potential treatment further in order to keep the side effects minimal. Use of β_2 agonists may therefore stimulate central release of key counter-regulatory hormones to aid quicker glucose recovery.

Role of Selective Serotonin Reuptake Inhibitors (SSRI)

Serotonergic neurons have a key role in the regulation of neuroendocrine function via both sympathoadrenal and hypothalamo-pituitary adrenal pathways. Although, SSRIs have been associated with hypoglycaemia, the hypothesis that blocking serotonin uptake might actually increase sympathetic outflow was tested in a 6 week trial of high dose fluoxetine (60-80mg) in non-depressed T1D individuals. Fluoxetine was reported to significantly increase both adrenaline (90% increase) and noradrenaline responses as well as increased endogenous glucose production (EGP) and lipolysis (all $p < 0.05$), (Briscoe, Ertl et al. 2008). However, Fluoxetine did not produce an increase in hypoglycaemia symptom scores, suggesting that the serotonergic pathways are not important to symptom generation, perhaps limiting their application clinically.

Role for theophylline

Theophylline, the adenosine-receptor antagonist is known to reduce cerebral blood flow, and can stimulate the release of catecholamines. Acute intravenous theophylline has been found to lower the glucose threshold (higher glucose level) for symptoms and adrenaline release during insulin induced hypoglycaemia in those with T1D and IAH (de Galan, Tack et al. 2002) . However when oral theophylline was given over a 2 week period, despite an increase in symptomatic awareness, there was no change in the magnitude of adrenaline release(Slonski 2013).

Manipulation of sensing mechanisms

Role of Fructose

Glucokinase (GK) plays an important rate limiting step in the phosphorylation of glucose to glucose 6 phosphate, (Efrat, Tal et al. 1994) which determines magnitude of insulin release and plays a role in brain glucose sensing during hypoglycaemia. Fructose 6 phosphate (F6P) inhibits glucokinase and fructose can be phosphorylated into F6P in the glucose sensing neurons. Based on this, Gabriely et al were able to demonstrate in T1D subjects that fructose delivered systemically produced a significant potentiation of the adrenalin response to hypoglycaemia with both an upward shift in its glycaemic threshold as well as increased magnitude of release. Fructose also augmented endogenous glucose production (EGP), which aided in improved recovery from hypoglycaemia. (Gabriely and Shamoon 2005)

Role for dietary supplementation

Medium chain fatty acids

The brain primarily relies on glucose for its main energy source, however, it does have the ability to use alternate fuels. Use of medium chain triglycerides (MCT), which can be rapidly metabolised into medium chain fatty acids, have been shown to ameliorate cognitive decline during hypoglycaemia. These can be used directly (as they can readily cross the BBB) and indirectly (via generation of ketones) to provide an effective alternate fuel source during glucose deprivation. Page et al, found that when MCT (three doses-20g, then 10g twice) were given at 25 minute intervals during induction of hypoglycaemia in patients with intensively treated T1D, there was a significant increase in free fatty acids and β -hydroxybutyrate. The MCT administration prevented the decline in cognitive performance (tests of immediate verbal memory ($p=0.009$), delayed verbal memory ($p<0.001$) and verbal memory recognition ($p=0.008$) during hypoglycaemia compared to placebo. However, reassuringly, MCT did not adversely affect the counter-regulatory response (Page, Williamson et al. 2009). Therefore it appears that MCT are preferentially used in cortical areas involved in cognition, without affecting the subcortical areas – such as ventromedial hypothalamus- which are involved with the generation of the CRR (Evans, Matyka et al. 1998).

Uncooked corn starch

Nocturnal hypoglycaemia is a significant problem to those with T1D accounting for >50% of all cases of severe hypoglycaemia. The night time period is the longest inter-prandial interval humans experience and in addition it is also a period of increased sensitivity to insulin and is associated with a reduction in counter-regulatory responses to hypoglycaemia.

Understanding the rate of blood glucose appearance following the ingestion of certain foods could help reduce nocturnal hypoglycaemia in T1D.

Many with T1D often take a bedtime snack to prevent nocturnal hypoglycaemia. However this only has an effect over the first half of the night. (Peak effect 2 hours). Uncooked corn starch, a slowly released carbohydrate, has a low glucose peak with a later peak (+ 4 hours) but can stabilise blood glucose levels for up to 7 hours. When given to those with T1D at night, blood glucose was found to be consistently 1.9mmol/L higher at 3 am compared to placebo ($p<0.01$) with also a significantly higher morning blood glucose (+1.1 mmol/L) (Axelsen, Wesslau et al. 1999). However, another study showed that when carbohydrate content was matched in the control arm, uncooked corn starch did not reduce frequency of nocturnal hypoglycaemia, although rate of decline in blood glucose overnight was slower (Ververs, Rouwe et al. 1993).

Caffeine

Caffeine appears to have good acute effects in improving neuroendocrine responses. However, chronic use can be linked with an increase in highly symptomatic episodes but with possible benefits of reduction of nocturnal hypoglycaemia. Caffeine has also been found to have beneficial effects on the cardiovascular system, with improved heart rate variability when given to patients with long standing T1D. (Richardson, Rozkovec et al. 2004) It is known that after a period of abstinence, acute caffeine ingestion can decrease cerebral blood flow (Debrah, Sherwin et al. 1996) and increase brain glucose utilization (Mathew 1986) and can also cause release of adrenaline (Nehlig, Daval et al. 1992). Caffeine ingestion 250mg (Debrah, Sherwin et al. 1996) or 400mg (Cox, Irvine et al. 1987) immediately prior to induced hypoglycaemia was associated with greater symptomatic awareness, and increased

levels of adrenaline. However a randomised placebo-controlled double-blind study (Watson, Jenkins et al. 2000) with 200mg caffeine (approx. 2 cups of drip brewed coffee) for 3 months, actually resulted in an increase in the number of symptomatic hypoglycaemia episodes (1.3 vs 0.9 episodes/week, $p<0.03$), although subjects experienced more intense warning symptoms (29 vs 26 total symptom score; $p<0.05$) during a subsequent hypoglycaemia clamp study. More recently, a double-blinded study (Reno, Daphna-Iken et al. 2013) reported that caffeine was associated with a significant reduction in duration of nocturnal hypoglycaemia (mean 49 vs 132 mins; $p=0.035$) assessed using continuous glucose monitoring.

Modulation of the renal haemodynamic

A recent single arm open label proof of concept trial (Perkins, Cherney et al. 2014) looked at empagliflozin (a sodium glucose co-transport 2 inhibitor) 25mg daily (for 8 weeks) in patients with T1D. Patients were advised to reduce bolus and basal doses by 30% as a safety measure. Despite a decrease in insulin dose (which was primarily basal) from 54.7 ± 20.4 to 45.8 ± 18.8 units/day ($p<0.0001$), mean A1C decreased from $8\pm 0.9\%$ (64 ± 10 mmol/mol) to $7.6\pm 0.9\%$ (60 ± 10 mmol/mol) ($p<0.0001$). Symptomatic hypoglycaemia (<3.0 mmol/L) also reduced from 0.12 to 0.04 events per patient per day ($p=0.0004$). All hypoglycaemic episodes (<3.9 mmol/L) were also reduced from 0.30 to 0.18 events per patient per day ($p=0.0001$) however the short duration of this trial limits further conclusions. In a recent dose finding exploratory study of 2 weeks duration, dapagliflozin showed a dose related reduction in glycaemic variability (greatest effect with 10mg- mean amplitude of glycaemic excursion (MAGE)), (-3.77 mmol/L (95% CI -6.09 to -1.45), however, the 95% CI did overlap with that of the placebo))(Henry, Rosenstock et al. 2015) suggesting that it may also be beneficial in reducing hypoglycaemia (although this was not specifically examined in the trial).

Modulating K_{ATP} channels in glucose sensing circuitry

It is now recognized that specialized glucose-sensing neurons in the brain play a key role in the detection and response to acute hypoglycaemia and that recurrent hypoglycaemia impairs the ability of these neurons to detect hypoglycaemia, contributing to the development of IHA. As in the beta-cell, SUR-1 selective K_{ATP} channels are a key component of this sensing mechanism and this suggest a potential role for K_{ATP} channel openers in the treatment of hypoglycaemia unawareness. Rodent studies have shown that diazoxide (6mg/kg) delivered directly to the hypothalamus or systemically amplifies the peak adrenaline response to hypoglycaemia, and therefore could potentially play a role in improving the protective counter-regulatory responses to hypoglycaemia.(McCrimmon, Evans et al. 2005). STUDY 1 of this thesis will examine whether we can use oral Diazoxide in a cohort of patients with longstanding T1D to magnify the counter-regulatory responses.

Incretins

So far the major focus on the use of incretins in T1D, has been as a result of GLP-1, being shown to reduce beta cell apoptosis, and also stimulate beta cell proliferation and improve survival in pre-clinical studies, therefore providing a potential adjunct therapy in early T1D. Furthermore, GLP-1 has also been shown to be a powerful suppressor of glucagon (Creutzfeldt, Kleine et al. 1996, Dupre, Behme et al. 2004), which may be particularly useful later on in the disease progression, as there appears to be a paradoxical increase in both fasting and post-prandial glucagon in T1D, which may contribute to a significant glucose load and excursions commonly seen in this patient group. GLP-1 infusion has thus shown a 45% improvement in post prandial glucose irrespective of C-peptide (Kielgast, Holst et al. 2011). There is evidence that reducing glucose variability can also reduce burden of

hypoglycaemia, however there is currently no evidence examining this concept, and will be examined in STUDY 2 of this thesis.

In summary, adjunct therapy in patients with Type 1 have been disappointing. Therapies such as use of amino acids and beta2 agonists may have improved outcomes during hypoglycaemia but have worsened overall glucose control. Agents that reduce GABA and theophylline have been shown to improve symptoms during hypoglycaemia but have not consistently improved the magnitude of counter-regulatory responses whereas agents such as the SSRIs, beta2 agonists and fructose have improved the adrenaline responses during clamp studies but this has not translated to a greater symptom profile. This dissociation between CRR hormone release and symptoms have been noted in a number of studies, suggesting generation of these components is through different mechanisms. It is also possible that because of the relatively small sample sizes in all the studies, that only large effect sizes would have been detected.

CHAPTER 3

STUDY 1 Use of K_{ATP} channel openers in Acute Hypoglycaemia

Background

One potential target for therapeutic intervention in people with T1D and IAH is the ATP-sensitive potassium (K_{ATP}) channel. This ligand gated ion channel is an octamer composed of 4 inward-rectifier potassium ion channels and 4 sulphonylurea receptor subunits (subtypes include SUR-1, SUR 2-A and SUR 2-B) and plays a critical role in transducing changes in cellular energy status into changes in action potential firing. Glucose sensing hypothalamic neurons thought to be central to the development of the suppressed hormonal counter-regulatory responses to hypoglycaemia (McCrimmon and Sherwin 2010), express SUR-1 containing K_{ATP} channels (Ashford, Boden et al. 1990, Dunn-Meynell, Routh et al. 1997, Kang, Routh et al. 2004). Following is a brief summary of where and how glucose is sensed, and how modulation of the K_{ATP} channel could be used in the improvement of the counter-regulatory responses in T1D.

Where is glucose sensed?

Plasma glucose concentrations are tightly regulated and normally maintained between 3.0 and 5.6mM, with an even tighter control present in the brain 0.5-2.5mM (Silver and Erecinska 1994). This is maintained through a complex network of glucose sensors and integrative networks.

Glucose sensors (GS) were first reported by Matschinsky (Matschinsky 1990) in the pancreatic beta cell. GS are responsible for detecting glucose concentrations in the extracellular fluid. (ECF). GS are defined as those that alter their action potential frequency

in response to changes in interstitial glucose levels. These neurons are unique, in that glucose is used both as a fuel, as well as identified as a signal that can regulate its activity. They are present in the gut, endocrine pancreatic cells, hepatic portal mesenteric veins and parts of the central nervous system (particularly in areas of the brain where the blood brain barrier is leaky or absent), with greatest representation in areas involved with regulation of neuroendocrine function, metabolism of nutrients and energy homeostasis. They are therefore represented primarily in the hypothalamus and the hindbrain (area postrema (AP), the nucleus of the solitary tract and the dorsal motor nucleus of the vagus) (Watts and Donovan 2010)) and ultimately are responsible for preventing wide glucose fluctuations, and therefore maintaining glucose homeostasis (McCrimmon 2008).

The hypothalamus has a number of areas which have been identified as key to glucosensing (GS) such as the arcuate nucleus, ventromedial hypothalamus (VMH), lateral hypothalamic area (LHA) and the dorsal medial hypothalamus (DMH)). (See figure 5) These regions appear to vary in the homeostatic and behaviours they regulate. For instance, the LHA has also been shown to be important in the feeding behaviour, and is a region that can co-ordinate metabolic and arousal state regulation through orexin (Ohno and Sakurai 2008). Therefore, it seems likely the different glucose sensing machinery may act to link glucose homeostasis to other bodily functions such as feeding behaviour and arousal.

Animal models can be invaluable in studying the molecular mechanisms involved in many of the processes involved in counter-regulation from glucose sensing to generation of defective counter-regulation. The counterregulatory responses have been studied in both human and animal models. For example, reduced hormonal counter-regulation following antecedent hypoglycaemia has been demonstrated in individuals with T1D, as well as in the rat (Powell, Sherwin et al. 1993) and mouse (Jacobson, Ansari et al. 2006) indicating a response to hypoglycaemic stress that is highly conserved.

A series of rodent experiments have illuminated the role of the VMH in the CRR to hypoglycaemia. Electrical stimulation of the VMH in a rodent model, mimics the CRR and activates the sympathoadrenal system (Stoddard, Bergdall et al. 1986), with local glucopenia induced by 2-deoxyglucose (a non metabolizable form of glucose) within the VMH having a similar effect (Borg, Sherwin et al. 1995). Both chemical destruction of the VMH with ibotenic acid and ample local perfusion with glucose obliterates the CRR to acute hypoglycaemia by 75% (Borg, During et al. 1994, Borg, Sherwin et al. 1997).

The VMH has therefore, been shown to be the main structure involved in brain glucose sensing in the hypothalamus and has a key role in the counter-regulatory responses. (Borg, During et al. 1994, Borg, Sherwin et al. 1995). It is a key region for interpreting peripheral signals of nutrient status. The VMH has been shown to have 2 distinct populations of neurons which respond to changes in blood glucose. These post-synaptic neurons can be classified into those which respond to increases in glucose (Glucose excited- GE) or those which respond to decreasing glucose levels (Glucose Inhibited-GI). Both are very sensitive to drops in extracellular brain glucose below 2.5mM, with minimal input when glucose rises above this level. There appears to be a synchronised activation of GI neurons and suppression of GE neurons as brain glucose drops from 2.5 to 0.1mM, which appears to initiate the neural network responsible for activating the sympatho-adrenal responses associated with hypoglycaemia (McCrimmon and Sherwin 2010).

The GE neurons have been identified in the ARC and the VMN. (Song, Levin et al. 2001, Song and Routh 2005). Most, but not all of the GE neurons share many features with the pancreatic beta cell in that, they appear to use the pancreatic form of glucokinase to sense glucose, and also contain the SUR1 isoform of the ATP sensitive potassium channel (K_{ATP}) as well as GLUT-2 (Schuit, Huypens et al. 2001). Glucokinase (GK) appears to have a key regulatory role in the sensing machinery of the GE neurons, demonstrated by the impairment

of glucose sensing when GK is downregulated (Kang, Dunn-Meynell et al. 2006). Tanocytes, specialized neurons that line the 3rd ventricle, have also been shown to have GK (as well as GLUT-2, KATP). Sanders et al discovered that selective inhibition of GK through alloxan delivery to the 3rd ventricle, taken up by the GLUT-2 in tanocytes, there was an increase in the CRR, suggesting that tanocytes are also crucial in facilitating the CRR response. (Sanders, Dunn-Meynell et al. 2004)

Early evidence that K_{ATP} channels play a role in glucose sensing in the physiological range, comes from the fact that concentration-response relationship between the action potential of VMH GE neurons and the K_{ATP} channel currents has a linear relationship for glucose concentration between 0.1 and 1.5mM glucose, and thereafter the slope of the curve decreases and plateaus up to 5mM glucose (Wang, Liu et al. 2004). It is thought to be the de-activation or opening of K_{ATP} channels in the GE neurons and subsequent activation of GI neurons which increase the sympathoadrenal responses to hypoglycaemia. (Kang, Routh et al. 2004, McCrimmon, Evans et al. 2005, Routh 2010)

GI neurons are predominantly involved when blood glucose levels drop. The first step may involve activation of the alpha 2 subunit of the AMPK. This turns on catabolic processes, which generate ATP while turning off anabolic ATP consuming processes. AMPK activation phosphorylates neuronal NO synthase (nNOS) leading to NO production in GI neurons. It has been shown that this nNOS is a critical step for the full generation of the CRR (Fioramonti, Marsollier et al. 2010). NO further activates AMPK via the NO receptor soluble guanylyl cyclase which increases cyclic GMP (cGMP). AMPK amplification via this method is needed for depolarisation of GI neurons in response to low glucose. Finally, this leads to closure of a chloride channel which many speculate could be cystic fibrosis transmembrane regulator (CFTR) (Murphy, Fakira et al. 2009) GI activation has been also shown to increase neurotransmitter release (McCrimmon, Shaw et al. 2008) associated with the initiation of the

counterregulatory response. A potential mediator of this response is thought to be GABA which is expressed in 56% of GE and 36% of GI neurons.(Kang, Routh et al. 2004). In acute hypoglycaemia, this inhibitory neurotransmitter decreases, potentially releasing the tonic inhibition of the GI neurons and leading to amplification of the counterregulatory response. This is demonstrated during in-vivo antagonism of VMH GABA (Chan, Cheng et al. 2008). Finally the VMN has projections to pre-autonomic networks present in the paraventricular nucleus of the hypothalamus (PVH) and the retrochiasmatic (RCH) area through which it is able to initiate the autonomic responses to acute hypoglycaemia (see figure).

The PVH is also thought to play a role in initiating the counter-regulatory response (CRR). Descending PVH (found in the lateral, dorsal and ventral) projections have been shown to regulate the pancreas and adrenal medulla, whereas the medial parvocellular part of the PVH drive the ACTH and glucocorticoid responses to hypoglycaemia. These signals along with signals from the LHA provide a direct way for the hypothalamus to interact with the autonomic motor components of counter-regulation.

There are also important glucose sensors present peripherally. These glucose sensors are present in the oral cavity, gut, portal mesenteric venous (PMV) system(Hevener, Bergman et al. 1997) and the carotid body (Heptulla, Tamborlane et al. 2001, Pardal and Lopez-Barneo 2002) (see figure 5). PMV neurons detect the glucose in the portal circulation before it enters the liver. The portal vasculature is richly innervated with afferents from both the vagus and spinal nerves. It has been proposed that the vagal and spinal nerves may be analogous to the functions of the GE and GI neurons, with the vagal signals (by promoting feeding) implicated in restoration of euglycaemia during hypoglycaemia. The signals are then relayed onto the higher brain centres, such as the hypothalamus (Watts and Donovan 2010).

There has been much debate as to whether these peripheral sensors contribute to the generation of the counter-regulatory response. Studies have shown that maintaining systemic hypoglycaemia in the context of euglycaemia in the porto-venous system by direct infusion of glucose into the portal vein (Donovan, Cane et al. 1991) diminishes the CRR. These neurons lie within the portal vein, and are mediated by spinal nerves (Hevener, Bergman et al. 1997). This finding was also recently confirmed by canine studies, showing that portal denervation also leads to blunting of the CRR during hypoglycaemia (Ionut, Castro et al. 2014). A study using rodent models demonstrated that the rate of glucose decline may determine whether peripheral or central sensors predominate (Saber, Bohland et al. 2008). Peripheral sensors namely the hepato-portal sensors have been shown to be responsible for nearly 90% of the CRR, when blood glucose levels drop slowly (Saber, Bohland et al. 2008). This may be of particular relevance when hypoglycaemia develops overnight, when the decline is slow and as a result of the basal insulin. However in the context of hypoglycaemia following meals or with exercise, where the decline is rapid, it may be the brain sensors that predominate in the sensing mechanism.

The other key part of the glucose sensing machinery is the glucose transporter. Glucose transporters are responsible for shifting glucose into and out of both the neuronal and astrocyte population. Circulating glucose enters the brain interstitium (without the need for insulin) through the blood brain barrier (BBB) predominantly via GLUT-1 (55kDa) which is expressed in the micro vessels of the BBB. (this is in contrast to adipose and muscle cells which need insulin for action of GLUT-4, its main glucose transporter) Glucose is then transported into neurons and astrocytes via predominantly GLUT-3 and GLUT-5 respectively. (Vannucci, Maher et al. 1997) During euglycaemia, the rate limiting step is the phosphorylation by the enzyme hexokinase 1, however the glucose uptake through the BBB, becomes the rate limiting step during hypoglycaemia. (Fanelli, Porcellati et al. 2004). There

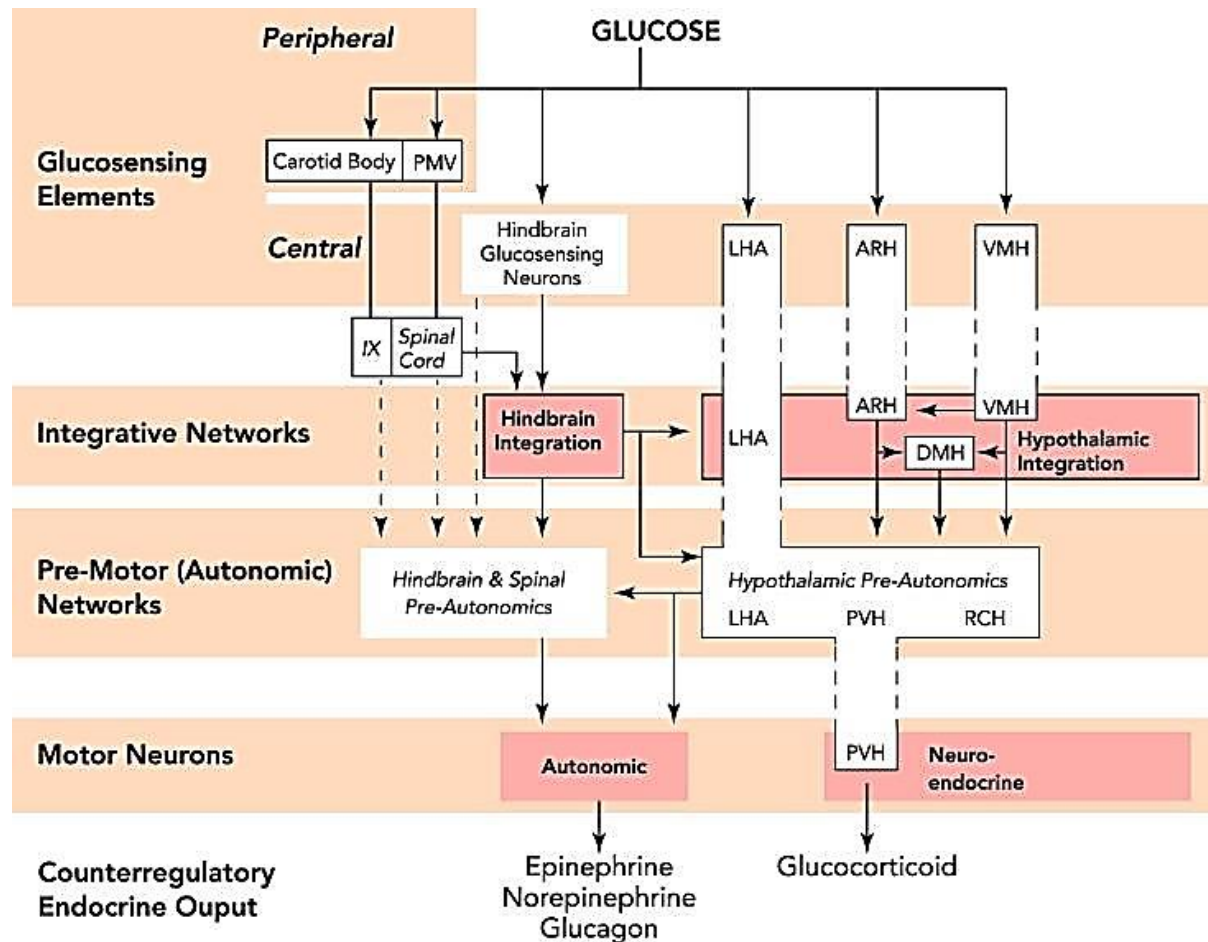
is also evidence now that GLUT-2(Marty, Dallaporta et al. 2005) (particularly in glucagon secretion) and GLUT-4(Puente 2009) are necessary for both glucose sensing and initiation of the CRR. Entry of glucose into the cell leads to phosphorylation by glucokinase which in turn causes a rise in the ratio of adenosine triphosphate to adenosine monophosphate levels (ATP: AMP).

However, during energy depletion, there is a resultant increase in AMP/ATP ratio that leads to activation of 5' AMP-activated protein kinase (AMPK). AMPK is a serine/threonine kinase that is activated during energy depletion and suppresses ATP consuming pathways and activates ATP generating pathways. AMPK within the hypothalamus is usually activated during fasting or central glucoprivation. It has been shown in rodent models that local in vivo activation of AMPK in the VMH amplifies the CRR while selective AMPK down regulation suppresses the CRR.(McCrimmon 2008)

Ultimately, the information collected through central and/or peripheral glucose sensors is then integrated at the central level, in the hypothalamus, hindbrain and autonomic ganglia, which are then responsible for effecting autonomic and neuroendocrine responses through to the pancreatic islets, adrenal medulla and the anterior pituitary resulting in a co-ordinated response.(Watts and Donovan 2010).

Figure 5; Diagram showing the neural network responsible for generation of the CRR system.

Shown are both the peripheral and central components that feed into hindbrain and hypothalamic integrative networks, which along with other inputs generate the autonomic and neuroendocrine responses. Taken from (Donovan and Watts 2014)



As the first study (STUDY 1) explores the therapeutic benefits of an agent that modulates the K_{ATP} channel, discussion of these channels in further detail will follow.

K_{ATP} channels

Cloning studies have revealed that the K_{ATP} channels consists of a large regulatory subunit (SUR) and small potassium subunits (Kir-) forming the channel pore (see Figure 6). (Aguilar-Bryan, Bryan et al. 2001) SURs are regulatory subunits which bind sulphonyureas. Their formation is controlled by the Sur1 and Sur2 genes that encode the high affinity SUR-1 and low affinity SUR2A and SUR2B respectively. SUR-1 is widely expressed in neuroendocrine cells and neurons whilst SUR-2A is found in heart and skeletal muscle; SUR-2B in smooth muscle. The pore subunits termed Kir-, or potassium inward rectifiers derives its name, because they are more efficient at transporting potassium into the cell and they come in 2 isoforms (Kir 6.1 and Kir 6.2). SURs preferentially bind to Kir 6.1 and Kir 6.2 to form large octameric channels. These are assembled in the Endoplasmic reticulum and the presence of a retrieval signal –RKR- ensures that only correctly assembled K_{ATP} channels are transported to the cell membrane (Zerangue, Schwappach et al. 1999). K_{ATP} channels are expressed within a number of cellular compartments (nucleus, sarcolemma, plasma membrane) and different tissues types, where the conformation of the channel largely determines its function. Five different types of K_{ATP} channels are currently known(Ashcroft and Ashcroft 1990). The best characterised K_{ATP} unit is the Type 1 channel and has been identified in a number of different cell types and are known to be calcium and voltage independent.

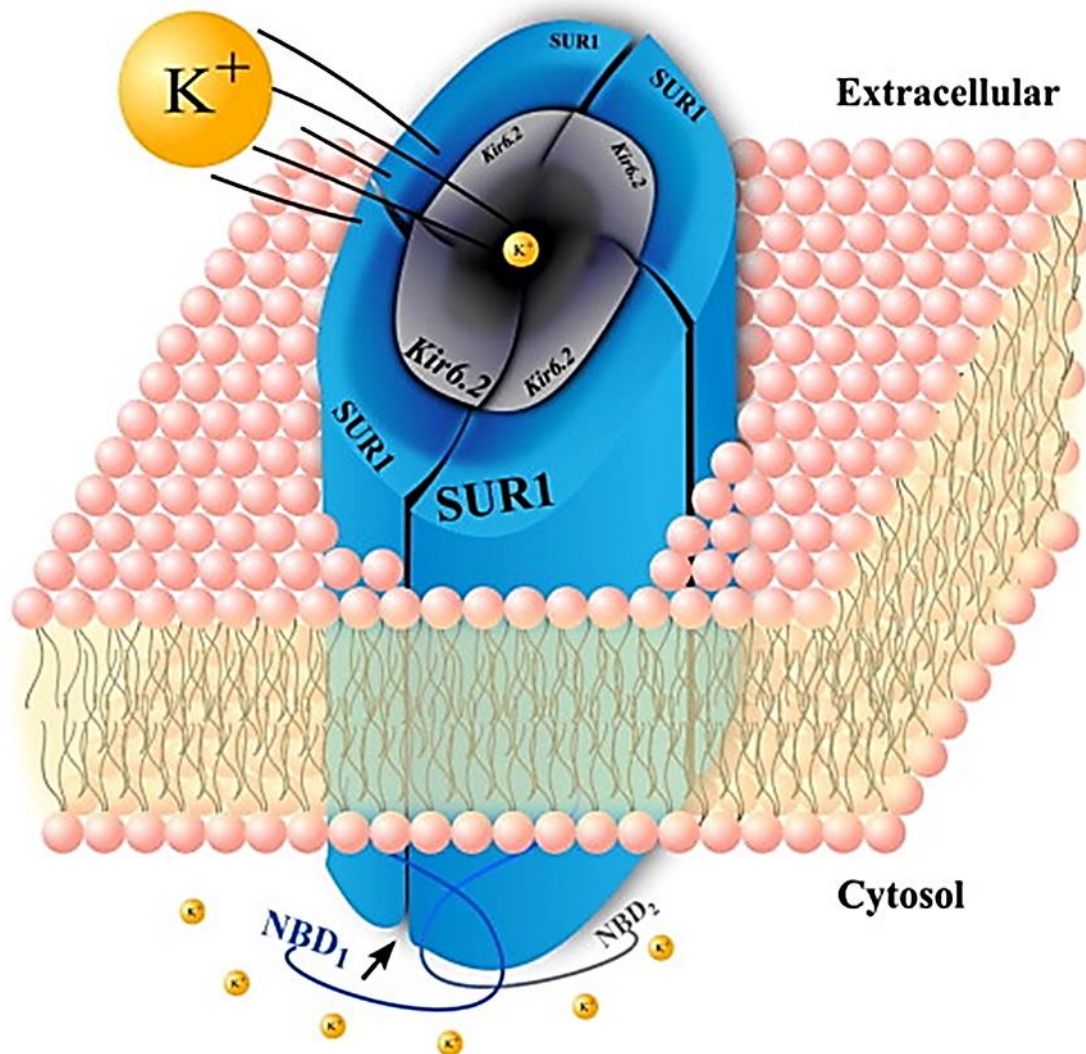


Figure 6; A schematic representation of the ATP sensitive potassium channel.

The Kir 6.2 subunits form the selective potassium pore, and are surrounded by the SUR1 subunits. This configuration of the KATP channels are found in both the pancreas and glucose sensing neurons. NBD1 and 2 are nucleotide binding domains. Taken from (Bonfanti, Alcazar et al. 2015)

K_{ATP} channels were initially located on the cardiac myocytes (Noma 1983) and the pancreatic beta cell (Cook and Hales 1984). In the pancreatic beta cell, these K_{ATP} channels are constantly open, leading to K⁺ efflux and hyperpolarisation, however, with increasing levels of glucose and ATP, this would then act to close these channels, leading to intracellular K⁺ accumulation followed by membrane depolarisation resulting in calcium influx and ultimately in insulin release in pancreatic beta cells.(Howell 1984). Further information about the K_{ATP} channel has been gained through the study of a mutation that affects the channel

such as occurs in Persistent Hyperinsulinemic Hypoglycaemia of Infancy (PHHI). In PHHI, there is uncoupling of the electrical activity of the cell to the metabolic status leading to permanent depolarisation of the membrane and an inappropriately high insulin secretion. (Aguilar-Bryan and Bryan 1999)

Activation (opening) of the K_{ATP} channels in the agouti related peptide (AgRP) expressing neurons has also been shown to be potentiated by insulin, which has also been shown to reduce gluconeogenesis, and reduce hepatic glucose production, contributing to the glucose lowering action of insulin (Pocai, Lam et al. 2005).

Therefore, glucose metabolism can be linked to the electrical activity of the beta cell through the K_{ATP} channel. K_{ATP} channels also exist in virtually all cell types including vascular smooth cells (Beech, Zhang et al. 1993) and glucose responsive neurons (Miki, Liss et al. 2001). The sensitivity of the K_{ATP} channel to the internal ATP concentrations vary between cells and act to couple metabolism to electrical activity. Ashford et al (Ashford, Boden et al. 1990) in 1989 showed in isolated neuronal cell recordings, that increased extracellular glucose and increased production of ATP, inhibited a K^+ channel and increased action potential generation. Therefore, in periods of low stress due to plentiful fuel, ATP closes the channel by binding to Kir 6.2, producing membrane depolarisation and increased electrical activity. In contrast, during periods of high stress such as during hypoglycaemia, Mg-nucleotide binding at the nucleotide binding domains (NBD) of SUR stimulates channel opening and hyperpolarisation. Agents such as cromakalim (an opener of the K_{ATP} channel) were shown to decrease the discharge of action potentials without altering their amplitude or duration in rodent neurons. (Finta, Harms et al. 1993)

Most glucose sensing neurons have mRNA for sulphonylurea receptor (SUR-1) and Kir 6.2 subunits. (Kang, Routh et al. 2004) Centrally, the K_{ATP} channel has been shown to be an

important link between glucoprivic feeding and the activity of the Glucose sensing neurons (GS). K_{ATP} channel activation (opening) sufficient to abolish activity of Pro-opiomelanocortin (POMC) neurons in the arcuate nucleus leads to hyperphagia and increased body weight (Plum, Ma et al. 2006). However partial activation which does not completely disrupt electrical activity has shown to blunt glucose sensing, and lead to impaired glucose tolerance (Parton, Ye et al. 2007). K_{ATP} channel activity has been shown to be regulated by lipids, such as PIP_2 and PIP_3 , which increase the likelihood of the channel being open, and decrease its responsiveness to ATP.

Decreased glucose has been shown to activate (open) K_{ATP} channels in the ventromedial hypothalamus, leading to glucagon and catecholamine release (Miki, Liss et al. 2001). Studies using electrophysiological recordings of both rat and mouse hypothalamic slice preparations and also local in-vivo application of K_{ATP} channel blocker (closer) have shown that closing the channel suppresses CRR while agents that open the K_{ATP} channel amplifies the CRR.(McCrimmon 2008) The importance of these channels were highlighted in rodent studies with deletion of the pore forming subunit of the K_{ATP} channel ($Kir 6.2^{-/-}$), which showed both lack of GR neurons in slices of VMH and a severely impaired counter-regulatory responses and abnormal feeding responses to hypoglycaemia. (Miki, Liss et al. 2001) In addition, K_{ATP} channels have been found to be important in modulating glucose uptake in muscle and in the central control of hepatic glucose output.

There also exists a common polymorphism in $Kir 6.2$ (E23K) which has predisposed individuals to Type 2 Diabetes (T2D). The functional effects of the E23K polymorphism are still controversial. Studies have shown both increases and decreases (Schwanstecher, Meyer et al. 2002, Riedel, Boora et al. 2003) in ATP sensitivity. A potential decrease in sensitivity would lead to an increased likelihood of an open channel.

In summary, during hypoglycaemia, direct detection of glucose by central glucose sensing neurons is integrated with afferent information from peripheral sensors, fed through the Nucleus Tractus Solitarius (NTS) to gauge systemic glucose levels. These hypothalamic and hindbrain neural networks then project to the paraventricular (PVN) nucleus of the hypothalamus. PVN activation is crucial in activating the main components of the CRR; the parvocellular neurons in the PVN activate the sympathetic nerve activation in the spinal cord, the DVN (dorsal motor nucleus to regulate the vagal parasympathetic efferent neurons) and the medial parvocellular of the PVN activates CRH which then activates the anterior pituitary to release corticotrophin (ACTH). (Reno, Litvin et al. 2013)

Direct *in vivo* local application of SUR-1 agonists to the ventromedial hypothalamus (VMH) has been shown to amplify (McCrimmon, Evans et al. 2005) while local SUR-1 antagonism suppresses (Evans, McCrimmon et al. 2004) the counterregulatory response to acute hypoglycaemia in rats. Moreover, systemic delivery of a SUR-1 selective agonist amplified counterregulatory responses during hyperinsulinemic-hypoglycemic clamp studies in normal and recurrently hypoglycemic rats, an effect that could be reversed by VMH K_{ATP} channel antagonism (McCrimmon, Evans et al. 2005, Fan, Ding et al. 2008). Based on these rodent studies, we hypothesized that therapeutic agents that activate SUR-1 selective K_{ATP} channels are likely to offer a potential means for restoring defective counterregulatory responses to hypoglycaemia in T1D.

The only licensed agent which is known to activate or open K_{ATP} channels is diazoxide and therefore we exploited this drug, to determine whether it could be used to potentiate the counter-regulatory responses to hypoglycaemia.

Diazoxide; a K_{ATP} channel opener

Diazoxide (C₈H₇ClN₂O₂S) was discovered through studies of the antibacterial effects of sulphonamides in the 1930s. This period of research into the active moieties of sulphonamides led to the synthesis of acetazolamide, the first useful diuretic, and subsequently led to the discovery of chlorthiazide. Diazoxide was initially developed as a non-diuretic hypotensive agent as a result of vasodilation (Rubin, Roth et al. 1962), an action for which it is still used clinically, before it was also noted to have a significant effect on raising blood sugars. More recently it has been shown to protect against cardiac ischemia due to its ability to induce ischaemic preconditioning (Oldenburg, Critz et al. 2003).

Diazoxide was previously, primarily indicated for the treatment of severe hypertension (particularly when associated with renal disease) (Standen, Quayle et al. 1989) and is still used for the treatment of chronic intractable hypoglycaemia occurring for instance in the context of an insulinoma (Trube, Rorsman et al. 1986). Usually given in a tablet form, Diazoxide is prescribed orally, and its use has been limited due to its multiplicity of effects which gives rise to a range of side effects including increased hair growth on the head, back, arm and legs, a change in taste, loss of appetite, stomach upset, diarrhoea, headache and tiredness.

Research in the last few years have suggested other potential uses for diazoxide including hypoglycaemia unawareness in type 1 diabetes, hyperlipidaemia and obesity and, perhaps more surprisingly, type 2 diabetes.

Pharmacology

Diazoxide is a benzothiadiazine derivative (7-chloro-3-methyl-2H-1, 2, 4-benzothiadiazine 1, 1 dioxide), that is closely chemically related to chlorthiazide. It was initially used for its

vasodilator and hyperglycaemic effects (in severe and refractory hypertension and insulinomas respectively)(1971). It is extensively protein bound (more than 90%) and is excreted by the kidneys. The plasma half-life following iv administration is 28.0 ± 8.3 hours. It appears that the maximal hypotensive effect occurs at about 5 hours after dosing which parallels the maximum blood levels(Pearson 1977). The primary mode of action of diazoxide is through the ATP-sensitive potassium (K_{ATP}) channels, which are a type of potassium channel gated by ATP and hence play a critical role as sensors of the metabolic status. K_{ATP} channels are composed of $K_{ir6.x}$ -type subunits and sulphonylurea receptor (SUR) subunits, along with some additional components. The $K_{ir6.x}$ - (either $K_{ir6.1}$ or $K_{ir6.2}$) subunits form the inward-rectifier potassium channel, while the SUR (SUR-1, SUR2A or SUR-2B) are transmembrane proteins that allow nucleotide modulation of the potassium channel.

Diazoxide exerts its effects through binding to the SUR subunit of the K_{ATP} channel which has the effect of 'opening' the channel leading to increased potassium entry into the cell and hyperpolarization of the cell membrane (inhibiting the generation of cell membrane potential). Sulphonyureas have the opposite action and once bound to the SUR subunit of K_{ATP} channels, have the effect of 'closing' the channel.

Pancreatic β -cells for instance, express SUR-1. Diazoxide binding leads to the K_{ATP} channel opening, and increases potassium membrane permeability leading to membrane hyperpolarisation, inhibition of calcium influx and diminished insulin secretory release. In contrast, cardiac, skeletal and vascular and non-vascular smooth muscle predominantly contain K_{ATP} channels of the SUR-2A sub-type. Diazoxide binding to this sub-unit leads to vasodilation, hence its use as a treatment for severe hypertension and is an explanation of the well-recognized adverse effects like reactive hyperaemia.

Trials of safety and efficacy

Diazoxide is a well-established treatment for congenital hyperinsulinemia and insulinoma and has been used in this context for over 40 years. Trials have been small, and have looked at the medical management of insulinomas as an adjunct to surgery or in those who are inoperable. To date there has not been a large randomised control trials looking at the efficacy of this treatment. Recent studies have suggested a number of possible novel indications for the use of diazoxide in obesity and both type 1 and 2 diabetes.

Obesity and Metabolic Syndrome

Studies of cell culture and animal models have demonstrated that diazoxide induces membrane hyperpolarisation in adipocytes, an action that suppresses lipogenesis and increases lipolysis through the regulation of key insulin sensitive enzymes. Diazoxide therapy in rats produces a reduced lipogenic state and results in lower levels of plasma leptin. In an open-label non-placebo controlled study of obese humans subjects, oral diazoxide when combined with a reduced calorie intake and increase physical activity resulted in additional reductions in both fasting and post-meal peak insulin levels by about 65% ($p < 0.001$), body weight by 9.4 kg ($p < 0.001$), waist circumference by -9.2cm ($p < 0.001$), diastolic blood pressure by 10.9mmHg ($p < 0.001$), and an increase in HDL ($p < 0.05$) (van Boekel, Loves et al. 2008). The results are comparable to currently available anti-obesity medications such as orlistat.

Type 1 and type 2 DM

A more recent development has been the study of diazoxide to prevent beta-cell decline in both type1 and type 2 diabetes. It is well recognized that preservation of beta-cell function in diabetes is associated with a reduced incidence of microvascular complications, such as retinopathy and nephropathy. Interventions that reduce beta-cell apoptosis therefore represent a potentially important therapeutic target in type1 and type 2 diabetes. There are a number of

on-going trials using immune-modulatory agents at diagnosis of type 1 diabetes to determine whether it might be possible to reduce disease progression. These agents are expensive and associated with frequent severe adverse events. Studies in animals have suggested that diazoxide might reduce beta-cell apoptosis by reducing the stress within the endoplasmic reticulum as well as through their action to hyperpolarise the plasma membranes and inhibit insulin release, both actions leading to 'beta cell rest'.

Bjork et al showed that supplementary diazoxide treatment at the onset of type 1 antibody positive adults for three months can preserve residual insulin secretion after a year. This is through initial suppression of insulin production providing a period of 'beta cell rest' which then preserves residual insulin secretion even after a year.(Bjork, Berne et al. 1996) The same group has also shown that inhibition of insulin secretion reduces autoimmune antigen expression.

Based on the similar hypothesis that beta-cell rest will preserve beta-cell function in the long-term, a proof of concept study in patients with type 2 diabetes showed that diazoxide given with night time insulin improved beta cell function, as demonstrated by the improved C-peptide glucagon tests, but there was higher post-prandial blood glucose in the morning. This can be explained through a selective action, as diazoxide prevents the insulin stimulating effects of glucose, but not that of glucagon. (Qvigstad, Kollind et al. 2004). Glycaemic control was not affected. Further human studies have shown that for long term glycaemic regulation, diazoxide should be given with insulin.

As a consequence of its non-selectivity of the SUR subunits within the K_{ATP} channel, it carries a side effect profile that could limit its use. Side effects seem to be limited to continuous use (multiple dosing daily) rather than intermittent use (once daily dosing).

Hyperglycaemia and oedema are the main dose limiting side effects, but these are usually reversible with dose adjustment (van Boekel, Loves et al. 2008).

The recent revelation of the mechanisms of diazoxide, i.e. K_{ATP} channel opening effects has opened up potential therapeutic roles for diazoxide as an option in diabetes. It may prove to be beneficial in the early stages to provide beta cell rest and it may potentially be used to improve awareness in those who have recurrent hypoglycaemia. The first study (STUDY-1) will seek to explore the therapeutic benefit of Diazoxide in patients with T1D (Bingham, Hopkins et al. 2003).

Two previous studies (Bingham, Hopkins et al. 2003, Raju and Cryer 2005) in non-diabetic patients failed to show an effect of diazoxide on the counter-regulatory response to hypoglycaemia. Both these studies delivered diazoxide to the subjects prior to induction of hypoglycaemia and gave diazoxide orally (5mg and 6mg/kg respectively). Diazoxide is highly protein bound (>95%) and so it is possible that the systemic levels reached were very low, although the drug would have been persistent given the long half-life (approximately 23 hours) of diazoxide. It is possible that K_{ATP} channel opening in hypothalamic glucose sensing neurons during moderate hypoglycaemia in non-diabetic individuals may already be near maximal and therefore this may be the reason for the lack of effect in the above human studies. By comparison, subjects with type 1 diabetes and IAH, it is likely that the hypothalamus has impaired glucose sensing and the K_{ATP} channel is therefore, less likely to be in the open-state and more likely to respond to K_{ATP} channel activators. To address this hypothesis we designed a study to specifically test the hypothesis that K_{ATP} channels were integral to the detection of hypoglycaemia in individuals with established T1D.

Methodology

This first study was a single-center, double-blinded, placebo-controlled randomized controlled trial (RCT). The study protocol, participant information sheets, informed consent forms were all approved by an independent research ethics committee (REC), Medicines Healthcare Products Regulatory Agency (MHRA) and also by NHS Tayside Research and Development (R&D). The study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained for each participant before inclusion in the study. Eligible participants were all adults between the ages of 18-60 who met the eligibility criteria. Inclusion and exclusion criteria are detailed below.

INCLUSION CRITERIA

1. Healthy adults (aged 18-55) with >5 years disease duration
2. On intensive insulin therapy (CSII or multiple daily injections)
3. HbA1C <8.0%
4. Ability to give written informed consent to participate in the study
5. BMI between 20-29

EXCLUSION CRITERIA

1. History of significant cardiac, hepatic, renal or neurological disease.
2. Significant head injury, epilepsy or hypoglycaemia-induced seizures.
3. Pregnancy.
4. Breast feeding mothers.
5. Participants on thiazide diuretics
6. Participants on other potassium channel openers (nicorandil, minoxidil)
7. Participants on medications with vasodilatory properties such as methyldopa, reserpine, theophylline and nitrites.
8. Participants on hydantoins (fosphenytoin, phenytoin)
9. Significant anaemia Hb<11.0 and Hct<33%.
10. If they have donated blood in the last 30 days.
11. All those who have participated in a clinical trial of an investigational medicinal produce (CTIMP) in the last 3 months
12. Participants who are already on diazoxide or who have a past history of allergy to diazoxide

Potential participants were identified using the Scottish Diabetes Research Network (SDRN), which is a register of patients who have pre-consented to be contacted for future clinical trials.

Other avenues of recruitment included approaching patients at diabetes clinics and through the various education programs offered for those with T1D. The study took place at the Clinical Research Centre, Ninewells Hospital, Dundee.

After initial screening which included collection of demographic information, each subject attended the Clinical Research Centre (CRC) on 4 separate occasions. On two of these visits, separated by at least 2 weeks the subject was given oral diazoxide or placebo before undergoing the hyperinsulinemic hypoglycemic clamp studies. Independent pharmacists dispensed either the active or placebo over-encapsulated capsules according to a computer generated randomization list. The Diazoxide and placebo were in capsule form and identical in appearance and pre-packed in bottles and given to the participant by the investigator prior to the clamp study. Both the participants and investigators were blinded to allocation of treatment.

The other 2 visits were to fit each participant with a continuous glucose monitor (RT-CGM), with low-glucose suspend (set at 4.5 mmol/l) where applicable, for 48 hours prior to each clamp study, to ensure that there were no significant hypoglycemic episodes ($<2.5\text{mmol/L}$), for at least 48 hours prior to each clamp study. Per protocol, the clamp was rescheduled if any sensor readings were less than 2.5mmol/L .

Real time Continuous glucose monitoring (CGM) as a tool for avoiding hypoglycaemia prior to clamp studies

CGM devices measure glucose via the glucose oxidase reaction in interstitial fluids and then translates this using a calibration reading into a plasma glucose value. The sensor is located on the tip of the needle. The Guardian Real-Time (Medtronic MiniMed, Northridge, CA, USA) device was used for these studies. The sensor was inserted using the auto insertion device. Due to the fact that antecedent hypoglycaemia over the previous 48 hours impairs

the counter-regulatory response to further hypoglycaemia and affects the glucose threshold for adrenaline release, it was important to employ a strategy designed to reduce all hypoglycaemia below 2.5mmol/L prior to the hyperinsulinemic hypoglycaemia clamp. The low glucose suspend (set at 4.5mmol/L) feature was also utilised to alert patients of their blood sugar and take appropriate corrective measures if necessary. Alarms at pre-set thresholds have shown to detect 90.1% of hypoglycaemia in subjects who wore the Enlite™ sensors (Medtronic Diabetes, Northridge, CA) (Keenan, Mastrototaro et al. 2012).

Assessment of Impaired Awareness of Hypoglycaemia

Several validated scoring systems exist. The most common of which are the Gold, Clark and Pedersen-Bjergaard (P-B) questionnaires. Gold method (Gold, MacLeod et al. 1994) has been used in these studies to assess awareness. It asks the question “Do you know when your hypos are commencing?” The participants respond by scoring on a 7 point Likert scale, with “1” for always aware and “7” for never aware. Greater than or equal to a score of 4 has been considered consistent with IAH. However, this method may lack sensitivity. For instance, individuals who do infrequent testing and have erratic control may not realise that they are experiencing hypoglycaemia. This was demonstrated in a CGMS study which identified 40-60% of all hypoglycaemic episodes which had gone unrecognised even in those who did SMBG regularly.(Cook, Bryzinski et al. 2013).

The Clark method(Clarke, Cox et al. 1995) contains 8 questions with much more detail on the symptomatic responses to hypoglycaemia as well as the subjects exposure to having moderate to severe hypoglycaemia with further exploration of glycaemic thresholds(Clarke, Cox et al. 1995). The questions explore symptom generation during biochemical hypoglycaemia (BH), frequency of moderate and severe hypoglycaemia and recognition of hypoglycaemia. A score of ≥ 4 is thought to be suggestive of IAH.

The (P-B) method asks the patient “can you feel when you are low?” with the possibility of 4 answers (always, usually, sometimes or never). Apart from always, all the other responses put the patients into the IAH category. This method is thought to be less selective and sensitive to clinical issues of importance.

The Gold and Clark methods show high correlation (r_s 0.866 $p=0.001$), whereas correlation is poor between these methods and the P-B method with the latter tending to estimate a much greater frequency of IAH (24, 26 and 62.5% respectively)(Geddes, Wright et al. 2007). It has been suggested that the P-B method is over-simplified, insensitive and unable to discriminate. However in the absence of any agreed gold standard measure except for the glucose clamp technique, no formal comparative study has been made.

The composite method is a further assessment tool, with six different diagnostic self-report criteria of IAH (change of symptoms, recognition, and threshold, experience of moderate and severe hypoglycaemia in the last year). Finally, the last criteria, which was the composite criteria detected IAH if a minimum of 3 out of the 5 previous criteria were positive(Janssen, Snoek et al. 2000).

A more detailed assessment of hypoglycaemia awareness, the Hypo A-Q questionnaire was recently developed and used in the HypoCOMPASS Study. This 18 item profile, asks patients to recall episodes of hypoglycaemia when awake and asleep. It also accounts for frequency of testing when feeling low, glycaemic thresholds and symptom generation amongst other parameters.

Assessment of hypoglycaemia awareness (HA) during a hyperinsulinemic, hypoglycaemic clamp study (and using a threshold of <3 mmol/L for autonomic symptoms) has been suggested as the gold standard in assessing HA. A study(Janssen, Snoek et al. 2000) looking at the agreement of various measures to the clamp study, showed that recognition criteria

(most similar to the Gold score) had a poor kappa 0.31, whereas the composite score had a greater agreement (κ of 0.49) with the clamp studies. There was also a very poor agreement with clamp threshold data and a patient hand held computer, where patients' would enter blood glucose estimations followed by home blood glucose monitoring (HBGM) and also degree of symptomatic awareness (κ of 0 with sensitivity and specificity of 50% each) .

Experimental stepped hyperinsulinemic hypoglycemic clamp.

Human counter-regulation can be studied robustly using the hyperinsulinemic hypoglycaemic clamp method, whereby an insulin infusion at a relatively high, constant dose is infused alongside a variable dextrose infusion.

The clamp technique was initially described by DeFronzo et al (DeFronzo, Tobin et al. 1979). It works on the principle that in the post absorptive state in non-diabetic individuals, the rate of endogenous glucose production (from liver 95% and renal 5%) matches whole body glucose utilisation, keeping the blood glucose in a tight physiological range. When exogenous insulin is added to this system, it suppresses endogenous glucose output, and increases whole body glucose utilisation, leading to a fall in the blood glucose. To prevent this fall, and maintain euglycaemia, a variable glucose infusion is started. The rate of this infusion must be sufficient to account for both the glucose flux into the glucose utilising cells and the reduction of glucose production. The hyperinsulinemic state induced by exogenous insulin must be sufficient to completely suppress the endogenous glucose output. Therefore the variable glucose infusion rate would be equivalent to the glucose utilisation into cells, in order to maintain euglycaemia.

Performing a glucose clamp requires two intravenous lines which need to be kept patent, one to deliver the insulin and glucose and the other to allow frequent blood sampling of arterialised blood. The 2 cannulae are usually placed in the antecubital fossa and the

contralateral hand. The insulin and glucose infusions go through the cannula in the antecubital fossa and need to run with finely tuned calibrated pumps that allow fine adjustment. The second cannula is placed into the contra-lateral hand and blood is drawn for glucose measurements. The hand is heated so that arteriovenous shunting occurs, which is most representative of the blood glucose “seen” by the brain. We used the arterialed sampling technique, which was done by placing the cannula into a hand vein, but then placing it in a heated box at 60 degrees C. This technique allowed heat induced vasodilatation and shorter blood transit time, and it has been found that the concentration of glucose and other metabolites is similar to that of arterial blood. (McGuire, Helderma et al. 1976) This technique eliminates differences seen in glucose commonly seen in arterial or even venous sampling that arise from variable insulin sensitivity in individuals. The arterialed blood sugar at a pre-determined level is “clamped” at a fixed pre-determined level. Glucose concentrations must be measured quickly, ideally within 1 minute, to allow adjustment of the glucose infusion. During each of the glucose nadirs, symptomatic, neuroendocrine and cognitive responses can be assessed.

The insulin infusion acutely raises the plasma insulin concentration to a hyperinsulinemic plateau for the duration of the clamp experiment. We used insulin infusion rate of 1.5mU/kg/minute. In order to accelerate the desired plateau of insulin concentration, we applied a priming dose of insulin (15u/hr) for all our studies during the euglycaemic period. This does lead to an initial overshoot of plasma insulin above the steady state achieved later in the clamp, however it does lead to shorter times to reach steady state. (DeFronzo, Tobin et al. 1979)

There have been many algorithms developed to calculate glucose infusion rates, but most of these have resulted in manual override to achieve required glycaemia as in our studies. A 20% glucose infusion is used and is preferable to a 10% glucose infusion, because of its

lower volume and to prevent over hydration. We found that the glucose infusion rates required to maintain euglycaemia increased within the first 40minutes from the start of the insulin infusion. This maybe as a result of the complete suppression of endogenous glucose production by insulin completely suppressing with a resultant increase in glucose utilisation.

Interstitial brain levels of glucose are thought to be 15-30% of the plasma glucose levels and this is consistent even in the hypoglycaemic range (Silver and Erecinska 1994). Plasma glucose was determined at 5 minutely intervals according to previously published literature. (Bergman, Finegood et al. 1985) Studies in non-diabetic humans, showed that it takes up to 20mins after the hypoglycaemic nadir (Evans, Pernet et al. 2000) before there is adequate generation of the counter-regulatory responses and therefore guided the design of our clamp experiment. Our clamp design, used a slow fall hypoglycaemia challenge, whereby each glucose nadir was maintained for 40mins during which CRR was assessed after the first 20mins and at the end of each nadir (to finish at 40mins). Symptoms and cognitive function testing was also done after 20mins and at the end of each nadir.

Thresholds are often calculated by a step wise multiple step clamp experiment. This, as in the first study (STUDY 1) drops the plasma glucose at regular intervals by a pre-specified amount and then the plasma glucose is clamped so that assessments such as counter-regulatory response, symptomatic and cognitive assessments can be made. This is continued until the lowest nadir is reached. Several methods have been described for calculation of thresholds with some investigators using baseline values (during the euglycaemic phase) to then calculate thresholds during the subsequent period of hypoglycaemia whilst others do two separate studies on two separate days; i.e. euglycaemic and hypoglycaemic studies. The latter ensures a reliable baseline for the above measures, from which thresholds are then calculated.

The evening prior to attendance, each participant was advised to reduce their night time long acting insulin by approximately 20% in order to avoid any overnight hypoglycemic episodes and advised to fast for at least 8 hours, and omit further short acting insulin, prior to coming to the Clinical Research Centre at 0800AM. On the morning of the clamp, a cannula was inserted into a dorsal hand vein of the non-dominant hand in a retrograde fashion and then placed in a heated box at 55°C to arterialize venous blood (Liu, Moberg et al. 1992). This line was used for blood sampling during the clamp study. In the contralateral arm, the ante-cubital vein was cannulated for insulin and glucose infusions.

Participants were given either oral diazoxide (7mg/kg) or matched over-encapsulated placebo in a double blind, randomized manner, 2 hours before the start of the euglycaemic plateau. The timing of oral Diazoxide ingestion was based on the available literature indicating that its hypotensive and anti-hypoglycemic effect lasted on average \approx 3-12 hours with a peak action at \approx 5 hrs. (Pearson 1977). Participants were initiated on 50ml/hr of insulin for priming purposes and this was continued until the blood glucose dropped to below 7mmol/L. Thereafter the intravenous infusion of insulin was continued at rate of 1.5mU/kg/min for the duration of the clamp. A variable dextrose infusion (Braun, Infusomat Space) (20% dextrose in saline), was started when the blood glucose dropped to below 7mmol/L, and this was adjusted every 5 minutes to reach the desired plateaus. Plasma glucose was brought down to between 4.0-5.0mmol/L over the first 2 hours. Euglycaemia was then maintained for the next 40 minutes. This was then brought down by 0.5mmol/L every 40 minutes until a blood glucose plateau of 2.5mmol/L was reached. The subject was blinded to their glucose level throughout the study. Following the completion of the hypoglycemic state, intravenous insulin was reduced to a third of the maintenance rate and iv glucose was given at 150% of the euglycaemic rate to enable quick recovery. Participants were given a meal and discharged when euglycaemia (>4 -

5mmol/L) was maintained. The clamp was then repeated at least two weeks later, to allow a sufficient wash out period for diazoxide.

Physiological measurements

Adrenomedullary adrenaline has been shown to be primarily responsible for the increase in pulse rate and cardiac output and for the widening of pulse pressure(Hilsted 1993). This appears to be a response to net vasodilation as a result of hyperinsulinemia and provides a compensatory mechanism to prevent hypotension. Blood pressure and pulse rate were measured every 10 minutes using an Accutorr Plus Monitor (Datascope Corp., Mahwah, New Jersey, USA).

Measurement of sympathetic nervous system

Several methods have been used to measure the outflow of the sympathetic nervous system (SNS), such as assessment of the heart rate and BP variability, microneurography, estimation of noradrenaline (NA)/adrenaline (A) spillover by isotope dilution and also through measurement of plasma noradrenaline/adrenaline. The spill-over technique only gives an indication of amount of NA released from the post-ganglionic sympathetic neurons. Most of the NA is subsequently used locally or re-uptake into axon terminals. There are also regional differences in sympathetic neural activity, which again complicates interpretation.

Single isotope derivative (radio enzymatic) methods used for the measurement of adrenaline have proved to be precise as well as sensitive and specific assays. However this measurement has a number of limitations. Plasma concentrations are dependent on both influx and efflux rates and rates of both these processes can vary. Most noradrenaline (NE) released by the postganglionic neurons may also never enter the systemic circulation, and be utilized locally.

Since NE is produced both by sympathetic neural and adrenomedullary activation, plasma levels will not discern its source.

Circulating noradrenaline is mainly produced from adrenergic post-ganglionic neurons, but under resting conditions, the SNS is the main (92-98%) source, with substantial greater increments in plasma NA on taking up an upright position (even in patients who are adrenalectomised). Hyperinsulinaemia also raises plasma NE (not plasma adrenaline), and also NA spillover (Paramore, Fanelli et al. 1998)...

Hypoglycaemia is a powerful stimulus for catecholamine release (Segel, Paramore et al. 2002) from the adrenal medulla, suggesting a suppression of SNS in the context of fasting or hypoglycaemia (Young, Rosa et al. 1984) (Cryer 1984, DeRosa and Cryer 2004). This was confirmed by a study comparing adrenalectomised patients with controls and found no adrenaline response in the latter group (DeRosa and Cryer 2004). Also, there is a poor correlation between A and NA, in the supine and standing position in healthy humans, however during hypoglycaemia, the correlation becomes highly significant ($r=0.829$, $p<0.001$), suggesting the adrenal medullae as the common source in this context.

Arterialized blood for insulin and counter-regulatory hormones (adrenaline, noradrenaline, and glucagon) were taken at midpoint and end of each plateau.

Laboratory assays.

Whole blood was measured at the bedside by a glucose oxidase method (Analox GM9D (Analox instruments, London, UK)). Samples were centrifuged to separate the plasma within 2 hours, and then stored at -80°C prior to assay. Hormones (Insulin-RIA-Diasorin; CV inter - 6.7%, intra - 5.8%), (Glucagon-RIA-MilliporeUK; CV inter 4.9%, intra 8.8%), (Adrenaline-EIA-Alpco; CV inter 22%, intra 16%), (Noradrenaline-EIA-Alpco; CV inter 16%, intra 22%)

were measured by ELISA, and samples were analysed in duplicate according to the manufacturer's instructions. Genomic DNA was prepared from whole blood using an Auto pure DNA preparation robot (Qiagen). Genotyping of rs5219 was performed by TaqMan based allelic discrimination (Thermo-Life Technologies) according to manufacturer's instructions.

Symptom assessment

Autonomic symptoms are generated by sympatho-adrenal activation, whereas the neuroglycopenic symptoms are provoked by cerebral glucopenia and cerebral dysfunction (Benzo 1983). Most of these symptoms are due to neuronally released transmitters rather than adrenomedullary catecholamines as most of the symptoms are lost by ganglionic blockade, cervical cord section (Pedersen-Bjergaard, Hoi-Hansen et al. 2007) and sympathectomy but not by adrenalectomy. Sweating and tremor (Fellows, Macdonald et al. 1986) in particular have both been shown to be well correlated with circulating concentrations of adrenaline.

We used the Edinburgh Hypoglycaemic Scale to assess symptoms during the clamp experiments. This scale was developed through partitioning the most commonly reported symptoms of hypoglycaemia reported by 295 insulin treated outpatients. This exercise revealed 11 key hypoglycaemic symptoms which were then categorized into three factors (autonomic, neuroglycopenic and malaise). These factors were validated on a separate group of 303 insulin treated patients (Deary, Hepburn et al. 1993) and showed good coefficients of congruence of the factors across the 2 groups studied. The scale measures the intensity of these 11 key hypoglycaemic symptoms on a 7 point Likert scale.

Subjects rated symptoms at the mid-point of every glucose plateau. Symptoms were scored on a validated questionnaire, the Edinburgh Hypoglycaemia Scale, scoring from 1 (not at all) to 7 (very severe) on a visual analogue scale. Symptoms included autonomic (hunger,

palpitations, sweating, shaking), neuroglycopenic (drowsy, confused, odd behavior, speech difficulty, incoordination) and non-specific symptoms (nausea, headache) (Deary, Hepburn et al. 1993)

Cognitive function tests.

A recent meta-analysis (Brands, Biessels et al. 2005) showed that specific cognitive domains are affected in patients with T1D. There is typically a mild-moderate slowing of mental speed and a diminished mental flexibility, with learning and memory (modalities which are susceptible to early brain disease) not being affected. The presence of microvascular disease was found to be of greater significance than hypoglycaemia exposure or glycaemic control, in determining the extent of cognitive dysfunction in the long term.

Numerous studies during acute hypoglycaemia have shown that during complex tasks, accuracy is often preserved, at the expense of speed (Strand, Anderson et al. 1934). In contrast to the effects of longstanding T1D on cognitive domains, acute hypoglycaemia impairs memory particularly working and delayed memory (Vindedzis, Marsh et al. 2013), attention, information processing, psychomotor function and spatial ability. The threshold for cognitive dysfunction is around 3mmol/L. One of the earliest studies (Maran, Lomas et al. 1995) showed that cognitive dysfunction (assessed through 4CT) occurs at a higher glucose level in those who were intensively treated compared to those who were on conventional treatment and non-diabetics respectively (3.2 ± 0.3 , 3.2 ± 0.2 and 3.0 ± 0.2 mmol/l, respectively ($p = \text{NS}$)), suggesting the reason for poor mental responses to hypoglycaemia in this group.

Insulin Induced Hypoglycaemia (IIH) has also been shown to affect several aspects of attention. It predominantly affects visual and auditory selective attention (ability to select information from one source, or of one kind rather than another) and attentional switching

(when attention is divided by 2 tasks), with preservation of sustained attention (vigilance or alertness). Psychomotor function and spatial ability have also been shown to deteriorate during IIH, although it appears to be less prominent in those with T1D than control subjects. A recent study(Graveling, Deary et al. 2013) showed that executive function (ability to plan, initiate, sequence, monitor and inhibit complex behaviours) was also impaired in the context of acute hypoglycaemia.

We selected 4 cognitive function tests which are known to be sensitive to the effects of acute hypoglycaemia and are easily repeatable in a short period of time.

Trail making B (Gaudino, Geisler et al. 1995) (TMB)

This is a test of motor speed and visual attention. Part A requires the subject to connect 25 consecutive numbers with lines, however part B, which was done as part of the battery of tests during hypoglycaemia requires the subject to alternate between numbers and letters. Part B is a more challenging task with greater demands on motor speed and visual search.

Digit Symbol Substitution (DSS)

This test derived from the Wechsler Adult Intelligence Scale(Wechsler 1981) is a test assessing the ability of the subject to code performed at speed. Total of 100 small blank squares arranged in 4 rows have a randomly assigned number symbol. A printed key on the opposite page, shows each symbol paired with a number. Both the DSS and TMB have been shown to be affected by moderate hypoglycaemia.

Digit span forward and backward(Wechsler 1981)

Digit span tests particularly assess verbal working memory (VWM) as confirmed by the activation of prefrontal cortical activities(Stern, Owen et al. 2000). The test involves the examiner reading out a progressive list of numbers, and the subject repeating the list either in

a forward or backward direction. The DSS has also been shown to have excellent internal reliability (0.70-0.90)(Conway, Kane et al. 2005)

4CT(Deary, Liewald et al. 2011)

This is a measure of reaction times. With greater discrimination during hypoglycaemia, we chose the choice reaction time D-L task, which requires the individual to make a response to one of a number of stimuli. This measure has been shown to be associated with measures of general fluid intelligence(Mortensen, Jensen et al. 2006) and also with survival over the next 15 years(Shipley, Der et al. 2006) . Internal consistency was also very high for the D-L task, with Cronbach's alpha to be 0.97 for correct responses.

These cognitive function tests were done in the same order starting at the midpoint of each plateau -Trail making B(TMB)(Kortte, Horner et al. 2002), Digit span backward(Dig-B)(1997), Digit symbol substitution test(DSST)(Wechsler 1981) and Four choice Reaction time (4CRT)(Deary, Liewald et al. 2011)

Data and statistical analysis.

The pre-specified primary endpoint was the magnitude of adrenaline responses at a glucose level of 2.5mmol/L. Secondary outcomes examined included, whether oral Diazoxide would affect glucose thresholds for activation of hormonal, symptomatic and cognitive responses or result in significant changes in heart rate or blood pressure. Data are presented as mean (SE). For the primary endpoint, normally distributed data were compared using paired samples t tests, while non-normally distributed data were compared using the Wilcoxon signed rank test. Statistical analyses were conducted using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com and $p < 0.05$ was considered

statistically significant. Repeated measures ANOVA was used to determine differences in other parameters measured over time, with t-testing used to localize effects where indicated.

Glucose thresholds for onset of symptoms, counterregulatory hormone responses and cognitive function were determined according to published protocols (Cranston, Lomas et al. 1994, Bingham, Hopkins et al. 2003). Glucose thresholds for onset of hormone responses were defined as the time of onset of a sustained (≥ 2 successive time points) increase in hormone concentrations ≥ 2 SDs above the mean of the two baseline measurements for that hormone (using individual values). Thresholds for the total symptoms were determined as the time at which the symptom score increased ≥ 2 over baseline on ≥ 2 successive time points. If no defined change occurred, then the lowest measured glucose was used as the threshold for that individual, in a similar fashion to other published literature. The glucose level at which there was a greater than 4% in the error rate was used to define thresholds of the Four Choice Reaction test.

Results

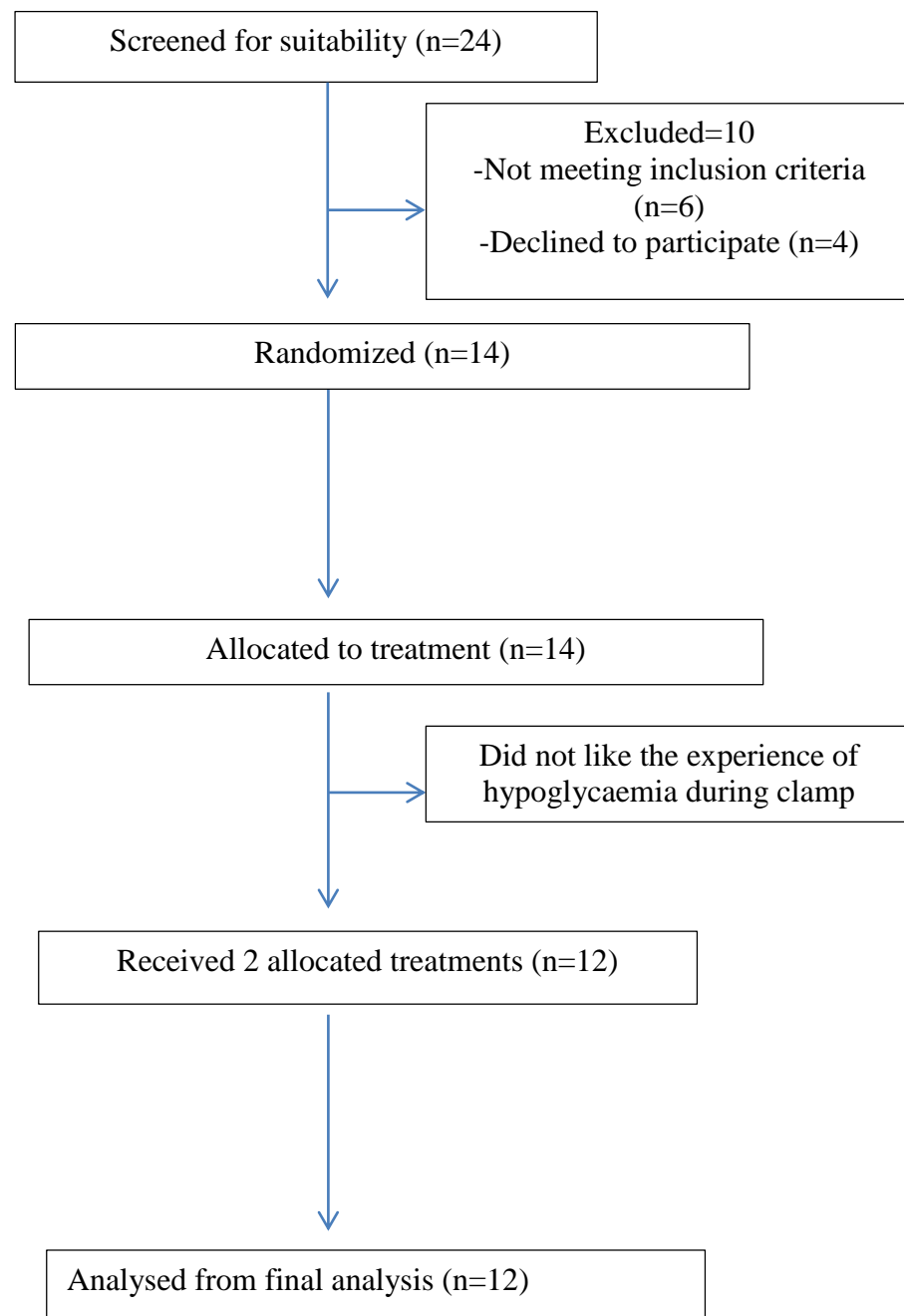
Participant characteristics.

Recruitment was from Jan 2012 to September 2012 (see Figure 1). Of the 24 participants screened, 6 did not meet the inclusion criteria and 4 withdrew consent; 14 subjects were randomized; 2 subsequently withdrew after the first clamp study. 12 participants (6 male and 6 female) completed all stages of the study (2 clamps). The median (range) age for this group was 43 (range 18-52). Duration of diabetes was 24(6-40) years and median HbA1c was 7.6%/60mmol/mol (6.9-8.0% (52-64mmol/mol)). There was an equal number of subjects on multiple daily injections (MDI) and those on continuous subcutaneous insulin infusion (CSII) therapy. 5 out of 12 participants had Impaired Awareness of Hypoglycaemia as classified by the Gold criteria (score ≥ 4) (Gold, MacLeod et al. 1994). However during the placebo clamp

studies, 11 out of 12 participants had an autonomic symptom threshold below or equal to 3mmol/L)(Geddes, Wright et al. 2007).

Figure 7; Consort diagram for STUDY 1.

Participation of subjects from initial screening to completion of studies. Once randomised subjects with long standing type 1 diabetes received either oral diazoxide or placebo in two hyperinsulinemic glucose clamp studies separated by at least 2 weeks.

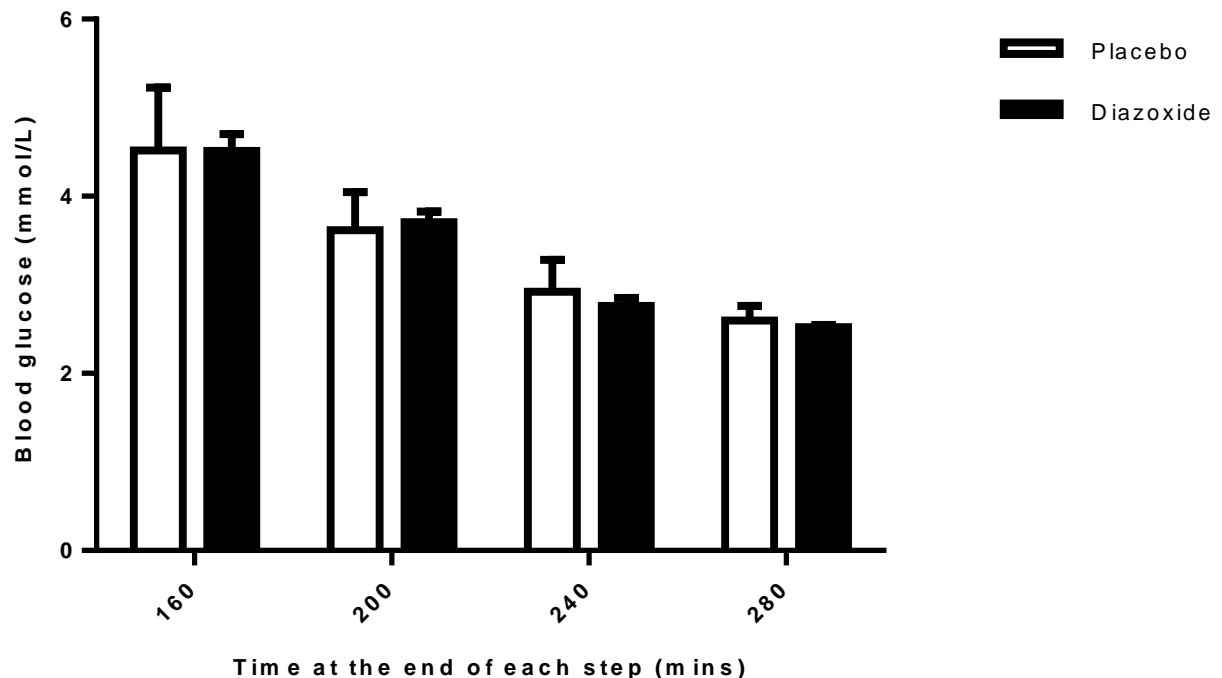


Hyperinsulinemic hypoglycemic clamp studies.

Mean (SEM) baseline blood glucose levels on the day of the hypoglycemic clamp studies for the Diazoxide (D) and Placebo (P) studies did not differ significantly between the two study days (10.6 (0.7) vs 11.8 (1.0) D vs. P $p=0.90$). Glucose levels during the two clamp procedures were also well matched.

Figure 8 Glucose profiles during stepped hyperinsulinemic hypoglycemic clamp studies.

The Hyperinsulinemic hypoglycemic clamp technique was utilized to slowly drop the blood glucose from euglycaemia (4.0mmol/L) to hypoglycaemia (3.5, 3.0, 2.5mmol/L). The drug (diazoxide or placebo) was given at 0mins, and after 120minutes, blood sugars were slowly dropped to euglycaemia during that time. Following which the clamp was commenced. Each nadir was achieved over 20mins, and then maintained for 20mins. Average blood glucose achieved at each of the desired steps during both the diazoxide and placebo clamp studies is shown in the bar chart below.



As expected, plasma glucose dropped with time (see Figure 2) over the stepped clamp (main effect of time $F(16,187) = 37.60$ $p < 0.05$). This drop was comparable in the two treatment

groups (main effect of treatment $F(1,187) = 0.2882$ $p = 0.59$, with no time X treatment interaction (time x treatment $F(16,187) = 0.4403$ $p = 0.97$). We maintained a mean insulin level of 79 (3.0) vs. 76 (2.8) mU/L (D vs. P) throughout the clamp period ($p = \text{ns}$).

Primary outcome

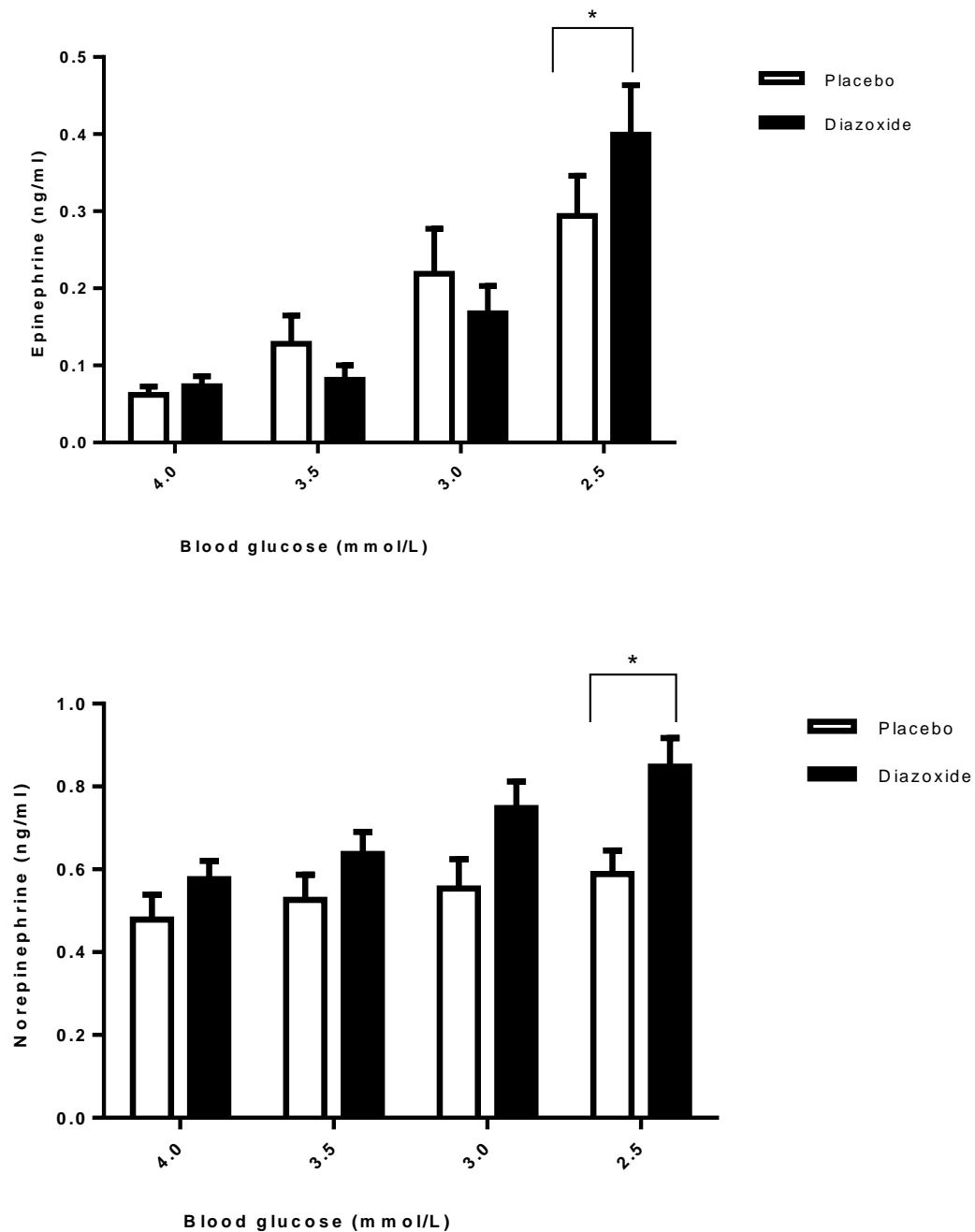
The primary outcome measure for this trial was the adrenaline response during the maximal hypoglycemic stimulus (2.5mmol/L). Baseline average adrenaline was 0.07 in both arms which rose to 0.29ng/ml and 0.40ng/ml in the placebo and diazoxide arms respectively. In support of our hypothesis, we found that following oral administration of Diazoxide, there was a 37% increase in mean (SEM) adrenaline responses (0.40 (0.06) vs. 0.29 (0.05) ng/ml, D vs. P; $p < 0.05$) and a 44% increase in mean (SEM) noradrenaline (0.85 (0.07) vs. P; 0.59 (0.06) ng/ml, D vs. P; $p < 0.05$) at plasma glucose of 2.5mmol/L. (See Figure 3)

Although glucagon levels remained, as expected, suppressed during hypoglycaemia, there was a non-significant trend towards higher mean glucagon levels in the Diazoxide arm (57.8 (11) vs. 50.0 (7.1) ng/l, D vs. P; $p = 0.21$). Consistent with the amplified catecholaminergic response to hypoglycaemia glucose infusion rates (GIR) required to maintain the hypoglycemic plateau were significantly lower different at 2.5mmol/L following oral diazoxide (71.6 ± 1.8 vs 77.5 ± 2.1 , D vs. P; $p < 0.05$).

Figure 9; Diazoxide amplifies catecholaminergic responses during acute hypoglycaemia in long standing Type 1 Diabetes.

(a) Plasma adrenaline and (b) plasma noradrenaline levels during baseline and each hypoglycemic plateau.

Placebo group shown as open bars, Diazoxide as closed bars. Values shown as mean (\pm SEM). * $p < 0.05$.



Despite the improved counterregulatory hormone response to hypoglycaemia participants experienced similar total symptom scores at an arterialized plasma glucose of 2.5 mmol/l following administration of Diazoxide (22 (3) vs. 19 (3), D vs. P; $p=0.32$). Similarly, the overall increase in autonomic symptoms following Diazoxide was not significant (10 (1) vs 9 (1) $p=0.26$). Cognitive performance of participants during the 2.5 mmol/l step was mixed (see Figure 4), with no significant impact of Diazoxide on Trail making B (30 (4) vs. 33 (5) s, D. vs. P; $p=0.65$), Digit symbol backward (6 (1) vs. 7 (1) , D. vs. P; $p=0.38$), or on 4 choice reaction time (547 (21) vs. 543 (18), D. vs. P; $p=0.82$), and a significant deterioration in Digit symbol substitution following Diazoxide (70 (9) vs. 81 (8); D. vs. P; $p<0.05$)

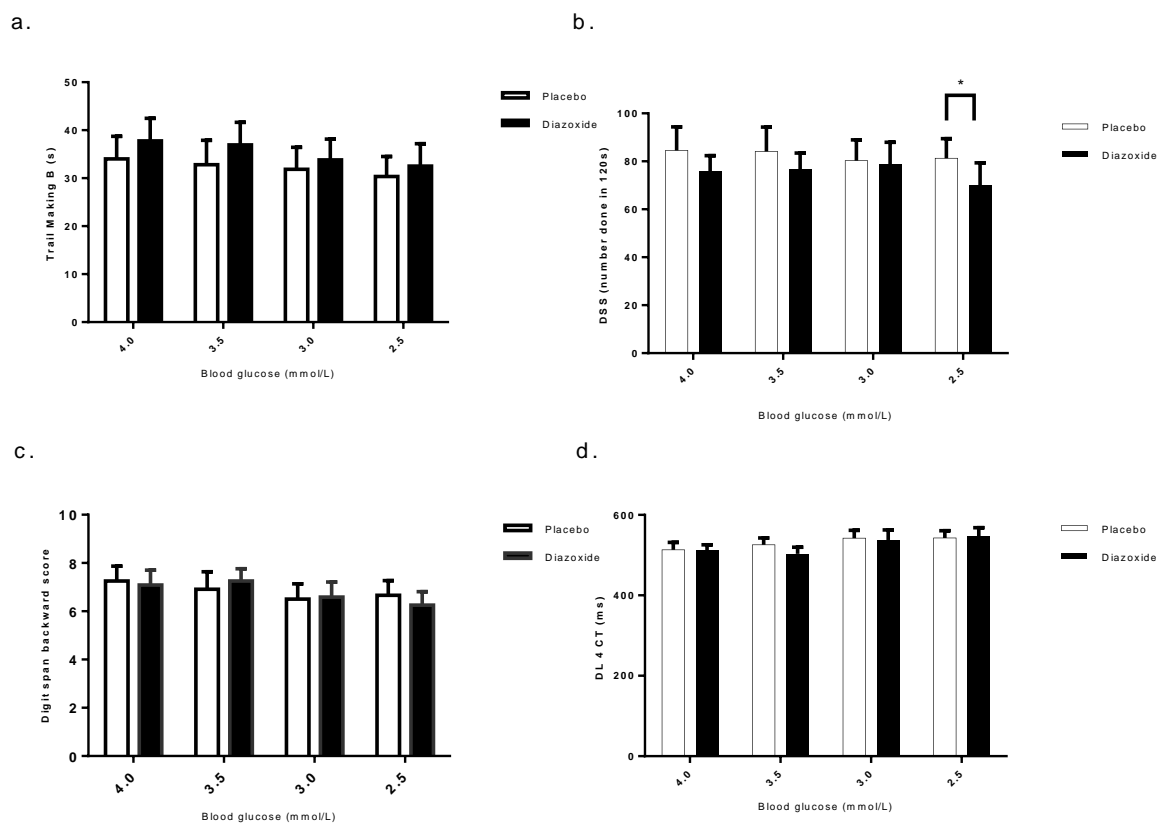


Figure 10; Cognitive function testing comparing Diazoxide to placebo at each of the glucose nadirs.

Of the 4 cognitive function tests performed, only Digit symbol substitution showed significant deterioration of performance with oral Diazoxide. (a) Trail making (b) Digit symbol substitution (c) Digit span backwards score (d) 4 choice reaction time scores are shown at baseline and at each hypoglycaemic plateau. Values shown as mean (\pm SEM). * $p<0.05$.

Secondary outcomes

Secondary outcomes in this study were to determine whether diazoxide would lower (higher glucose for initiation) the glucose thresholds for onset of hormone responses. Thresholds were defined as the time of onset of a sustained (≥ 2 successive time points) increase in hormone concentrations ≥ 2 SDs above the mean of the two baseline measurements for that hormone. Although the glucose threshold for generation of both adrenaline and noradrenaline responses to hypoglycaemia were lower following administration of diazoxide these did not reach significance. (See Table 4)

Table 4 Glucose thresholds for the key counter-regulatory hormones and symptoms for the Diazoxide study.

The glucose threshold defined as the glucose level at which there is a sustained (>2 successive Time Points) increase in hormone concentrations or symptom scores above the mean of the two baseline measurements for that hormone or symptom. Values reported as mean \pm SEM.

| Glucose Thresholds (mmol/L) | Placebo | Diazoxide | P value |
|-----------------------------|---------------|---------------|---------|
| Adrenaline | 3.1 \pm 0.4 | 3.2 \pm 0.3 | 0.75 |
| Noradrenaline | 2.8 \pm 0.5 | 2.9 \pm 0.4 | 0.49 |
| Glucagon | 2.8 \pm 0.5 | 2.6 \pm 0.3 | 0.24 |
| Symptoms | | | |
| Autonomic | 2.7 \pm 0.3 | 2.8 \pm 0.4 | 0.39 |
| Neuroglycopenic | 2.8 \pm 0.4 | 2.8 \pm 0.4 | 0.17 |

Genetic analysis- E23K polymorphism

The E23K polymorphism in the K_{ATP} channel results in an increase in the likelihood of the K_{ATP} channel being open in the resting state (Schwanstecher, Meyer et al. 2002, Hamming, Soliman et al. 2009) and influences individual responses to sulphonyureas (Javorsky, Klimcakova et al. 2012). To determine whether the E23K polymorphism might influence individual responses to Diazoxide all 12 participants were genotyped and the cohort divided

into diazoxide-responders and diazoxide-non-responders. A diazoxide-responder was defined as an individual who had a greater than double the standard error of the mean increase in adrenaline responses at 2.5mmol/L following diazoxide. In our study cohort, 7 of the 12 participants (58%) carried the K23 allele (2-KK, 5-EK), while the rest were of the wild type homozygous E23. Intriguingly, participants who expressed only the wild type E23 allele were all diazoxide-responders (see Figure 6), while those hetero- or homozygous for the K23 allele were significantly less likely to respond to Diazoxide (Pearson's chi squared, $\chi^2=6.12$; $p=0.013$) (see Figure 6). Those who expressed the wild type E23 allele also showed greater magnitude of adrenaline response particularly as the blood glucose dropped down to 2.5mmol/L (see Fig5).

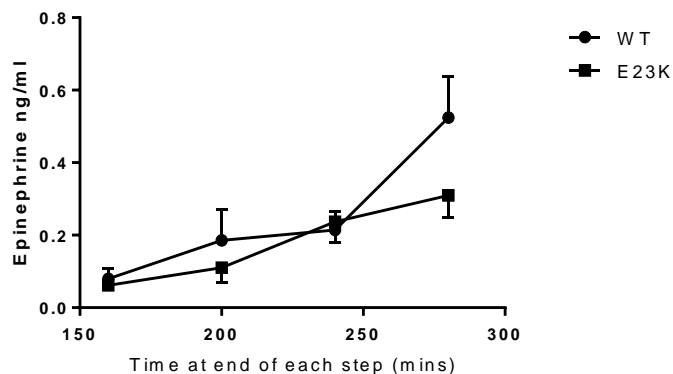


Figure 11 Epinephrine (adrenaline) response at the end of each glucose nadir.

This shows that there was fairly minimal adrenaline responses up to 250mins (achieving 3.0mmol/L plasma glucose), but responses diverge as the blood glucose drops down to 2.5mmol/L. Closed circles represent those with the wild type (WT) homozygous E23, and closed boxes are those with the K23 allele.

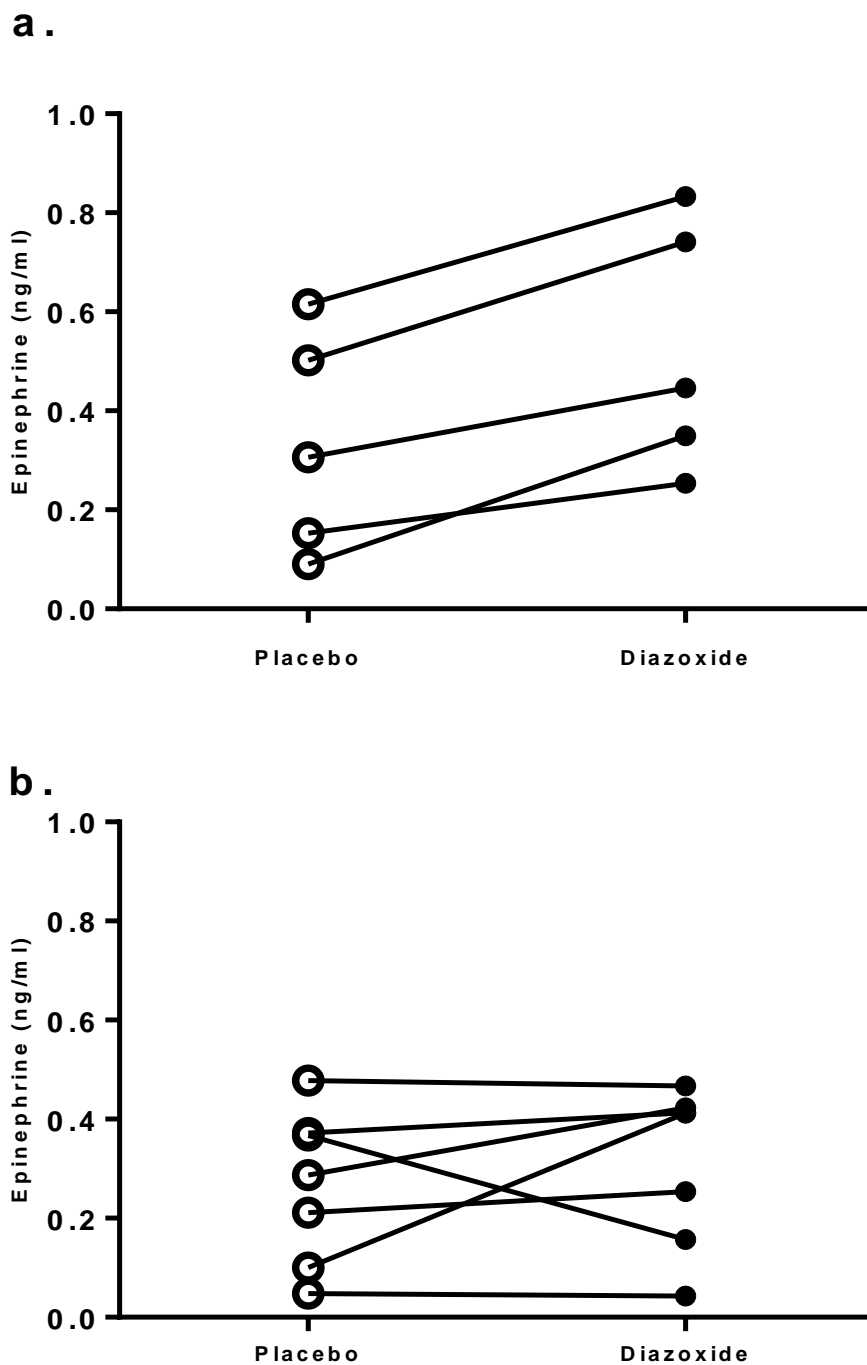


Figure 12 Epinephrine responses depending on genotype

E23K polymorphism in Kir 6.2 predicts response to Diazoxide during acute hypoglycaemia. This figure shows the magnitude of the adrenaline response during acute hypoglycaemia (2.5mmol/L) following placebo or diazoxide in individuals who expressed (a) Wild type (EE) or (b) Homo or heterozygous (KK,EK) for this E23K polymorphism for the Kir 6.2 channels. Results for each individual under the two conditions are shown.

Adverse events. Systolic BP was comparable between the two groups, with no effect of treatment (main effect of treatment $F(1, 22) = 0.001228$ $p=0.97$). Similarly there was no effect of treatment on Diastolic BP (main effect of treatment $F(1, 22) = 0.4602$ $p=0.50$) or on pulse rate (main effect of treatment $F(1, 22) = 2.893$ $p=0.10$) see figure below.

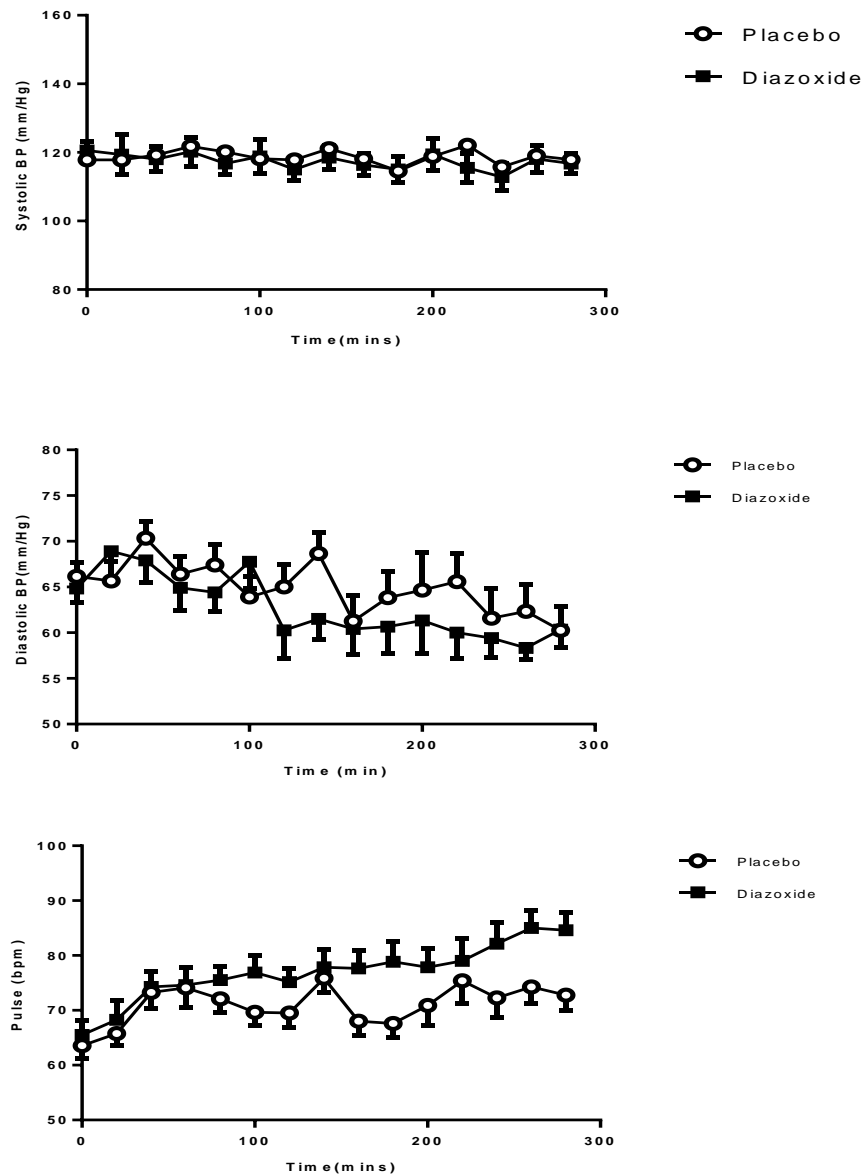


Figure 13; Haemodynamic responses during the clamp studies.

There was no significant change in any of the haemodynamic parameters during the clamp studies. The figure shows (a)systolic BP (b)diastolic BP(c)pulse rate from time 0 (drug given) to the conclusion of the clamp studies. Open circles represent the placebo arm, and closed circles are the diazoxide arm.

One participant had nausea and vomiting which was short lived in the recovery phase of both Diazoxide and Placebo studies, and reported nausea as being one of her usual symptoms during hypoglycaemia. One participant had nausea and a bout of vomiting in the recovery stage after receiving Diazoxide. There were no serious adverse events/reactions.

Discussion

In the current study, we demonstrate for the first time in individuals with type 1 diabetes of long duration that oral diazoxide (7mg/kg) given prior to acute hypoglycaemia can significantly increase the magnitude of both adrenaline and noradrenaline counterregulatory responses. Moreover, we make the novel observation that the E23K polymorphism in the Kir6.2 subunit of the K_{ATP} channel predicts response to diazoxide therapy during hypoglycaemia in T1D

The importance of K_{ATP} channels to hypothalamic glucose sensing was first proposed by Mayer (Mayer 1953), and the critical role of K_{ATP} channels in hypothalamic glucose sensing has since been demonstrated in cell culture models (Beall, Hamilton et al. 2012), *ex-vivo* hypothalamic slices (Ashford, Boden et al. 1990), transgenic mouse models (Miki, Liss et al. 2001) and following local pharmacological agonism or antagonism of hypothalamic K_{ATP} channels in the rat (Evans, McCrimmon et al. 2004, McCrimmon, Evans et al. 2005). More recently, the potential utility of K_{ATP} channel openers to improve counterregulatory responses to hypoglycaemia has also been demonstrated in rodent models of type 1 diabetes and defective counter regulation. (Fan, Ding et al. 2008).

The mechanism by which diazoxide improves the neuroendocrine response remains unclear. While the *in vitro* and *in vivo* rodent literature support a central action of K_{ATP} activators, all glucose-sensing cells both centrally and peripherally have been shown to contain Kir 6.2 and Sur-1 components of the K_{ATP} channel. It is therefore possible that oral diazoxide acts primarily through peripheral K_{ATP} channels, such as those in the hepato-portal veins (Saberli, Bohland et

al. 2008). The comparatively 'slow-fall' in glucose with the multi-step clamp would also be consistent with activation through hepato-portal sensors (Saber, Bohland et al. 2008). In contrast, studies of direct hypothalamic modulation of K_{ATP} channels in rodents during hypoglycaemia (Evans, McCrimmon et al. 2004, McCrimmon, Evans et al. 2005) support a central action of diazoxide, as do the findings in both our own study and that of Bingham et al. (Bingham, Hopkins et al. 2003) of an effect of diazoxide on tests of psychomotor speed in human subjects. In a related study, Kishore et al. (Kishore, Boucai et al. 2011) demonstrated in the rodent model that the extra-pancreatic action of diazoxide to suppress hepatic glucose production could be reversed through ICV delivery of the K_{ATP} channel blocker, glibenclamide. Moreover, this group performed time course studies and were able to detect diazoxide in the CSF of rodents rapidly after oral ingestion reaching levels of 0.26 ± 0.06 $\mu\text{g/ml}$ 1 hour after gavage and 0.78 ± 0.03 $\mu\text{g/ml}$ by 4 hours, providing convincing evidence that diazoxide penetrates the blood-brain-barrier (BBB) (Kishore, Boucai et al. 2011). Species differences may effect BBB permeability to Diazoxide, however Diazoxide contains an ionisable sulphonyl group making it extremely lipid soluble and therefore able to partition into the lipid bilayer and it has a pKa of 8.5, a favorable constant, for penetration through the BBB (Kamp, Kizilbash et al. 2003). Overall, the favorable pharmacological properties of Diazoxide as well as the human and rodent *in vivo* studies to date provide support for the hypothesis that its effect to amplify the counterregulatory response to acute hypoglycaemia is through a direct action on key hypothalamic glucose sensing regions.

Our findings contrast with those of Bingham et al. (Bingham, Hopkins et al. 2003) and Raju et al. (Raju and Cryer 2005) who failed to see significant effects of oral diazoxide on counterregulatory responses to hypoglycaemia in non-diabetic subjects. However in the study Bingham et al. (Bingham, Hopkins et al. 2003) did report that hypoglycaemia-induced peak adrenaline levels were higher following diazoxide (adrenaline 7.37 ± 1.89 vs. 6.18 ± 2.28 nmol/l,

respectively, $p=0.055$). Similarly, although Raju et al. do not provide actual values to compare adrenaline and noradrenaline responses during the latter stages of the mild hypoglycaemic challenge (3.0mmol/L), these appear greater in those subjects given diazoxide (Raju and Cryer 2005). Both studies used lower doses of diazoxide (5 and 6 mg/kg, respectively) which may contribute to their failure to see a significant effect. In addition, it is possible that K_{ATP} channel opening in hypothalamic glucose sensing neurons during moderate hypoglycaemia in non-diabetic individuals may already be near-maximal. By comparison, subjects with type 1 diabetes and IAH, the hypothalamus may have impaired glucose sensing and the K_{ATP} channel is therefore, less likely to be in the open-state and more likely to respond to K_{ATP} channel activators. Consistent with this hypothesis, even within our own study cohort responsiveness to Diazoxide was significantly reduced by the presence of the K23 allelic form of the Kir6.2 subunit which predicts an increased likelihood of the K_{ATP} channel being in the open state.

In this present study oral Diazoxide was able to augment the counterregulatory response sufficient to significantly reduce requirements for exogenous glucose during the clamp procedure suggestive of a real impact on whole body responses. Despite this we did not see a statistically significant change in glucose thresholds for counter regulation or for overall symptom responses. However, the subjects in this study had diabetes of relatively long duration and despite only 5 of the 12 participants having IAH as defined by Gold criteria, 11 out of 12 participants had an autonomic symptom threshold below or equal to 3mmol/L during the clamp studies. However, the above questionnaires rely on the patient having an insight into their lack of hypoglycaemia awareness. These scores therefore could be an underestimate of the degree of awareness, and as they are often used in the literature to quantify the problem. Indeed the studies so far have asked the patient to SMBG at least 4 times a day, and then if the patient's results have been less than 3mmol/L, they have been asked to complete the Edinburgh Hypoglycaemia Scale (EHS). However, in the majority

most patients would not be testing their blood glucose 4 times a day every day, and so assessment of hypoglycaemia in these patients is entirely reliant on symptomatic awareness (and not on SMBG). There have been no studies looking at the agreement between the Gold score and routine BG monitoring as determined by the patient, and not enforced by a clinical trial.

In this study, all subjects had a profound defect in symptom and hormonal counterregulatory responses to hypoglycaemia. It is likely that our study would only have detected large effect sizes in these secondary outcomes. In addition, a limitation of our study is that we calculated thresholds based on the euglycaemic period prior to the induction of hypoglycaemia as reported by others (Leelarathna, Little et al. 2013). An additional euglycaemic control arm to the study would have both reduced baseline variability and controlled for effects of time dependent changes.

Individuals with IAH often have long-standing type 1 diabetes, higher thresholds (first significant increase at a lower glucose level) for hormonal and symptom counter regulation, and a higher risk of severe hypoglycaemia (Cryer 1994, Mokan, Mitrakou et al. 1994). The higher threshold for symptom generation in particular is viewed as being a major risk for severe hypoglycaemia in that loss of usual warning symptoms means that hypoglycaemia when experienced is likely to be more severe and less likely to lead to appropriate corrective behavior. A clear goal of therapeutic intervention is therefore to restore thresholds for hypoglycaemia symptom generation. In this study, and consistent with others (Bingham, Hopkins et al. 2003), diazoxide did not significantly affect threshold for hypoglycaemia generation or for counterregulatory hormone responses. Despite this, following diazoxide activation of symptom and counterregulatory responses all tended to be initiated at higher glucose levels and it is likely that our study would only have detected large effect sizes.

It can be argued that the background noise is very different between real life and during clamp experiments. Patients are often fasted from midnight before the day of the clamp, as a result, the presence of hunger was often not a discernible symptom specific to hypoglycaemia. Subjects are supine for the duration of the clamp, and therefore discerning neuroglycopenic symptoms may be challenging for the subjects. It is possible that symptoms are more noticeable to them in the context of activity rather than when they are supine as in this study. Our study also had a slow fall in glucose and our lowest nadir was only 2.5mmol/L (chosen for ethical reasons). The slowness of the glucose fall and the lack of activity, may have implications in accurately determining the neuroglycopenic symptom complex experienced by the individual during the studies.

The order in which tests are done could also influence symptoms. The glycaemic threshold for the symptoms could also as suggested previously (McCrimmon, Deary et al. 2003), be influenced by the cognitive tasks undertaken. In this study, symptoms were assessed using the EHS, before assessment of cognitive function. In retrospect, performing the cognitive function tests prior to symptom assessment, may elicit greater neuroglycopenic symptoms particularly in this group of subjects with IAH, where symptoms are subtle.

However another study (Suhaimi, Le Compte et al. 2010) looking at symptom generation during insulin induced hypoglycaemia suggested a good allocation of symptoms to either autonomic or neuroglycopenia symptoms. In those who had neuroglycopenic symptoms as their initial symptoms, the overwhelming initial symptom in 82% of subjects was an inability to concentrate. However the study protocol was different from STUDY 1, in that all subjects tested retained good awareness of hypoglycaemia, the method of induction of hypoglycaemia was through the insulin infusion technique where the fall of glucose was rapid ($0.10 \pm 0.005 \text{ mM/min}$) and plasma glucose levels were lower (1.3-2.0 mM/L) potentially capturing the neuroglycopenic threshold for symptom generation. A meta-analysis of 9

separate studies (McCrimmon, Deary et al. 2003) (all in control subjects and T1 with good hypoglycaemia awareness) however showed that the method of induction (insulin infusion vs hyperinsulinemic glucose clamp) had a close agreement with reported hypoglycaemia symptoms. In conclusion, it may be that in a cohort of subjects as in our study who in the majority had IAH (11/12), that a lower induced glucose nadir and performance of cognitive tasks prior to symptom assessments may elaborate greater neuroglycopenic scores, and allow greater ability to distinguish between treatments (D v P).

An interesting further finding in the current study was the effect of Diazoxide on the DSS task. This psychomotor task is often used in hypoglycaemia studies and provides a robust and sensitive measure of cognition, despite there being no clear agreement on the cognitive processes engaged by this task (Peeters, Nicolson et al. 2004). Bingham et al, (Bingham, Hopkins et al. 2003) reported that non-diabetic subjects showed a significant prolongation on the 4-choice reaction time (4CRT) task during hypoglycaemia following diazoxide, but no effect was seen on Stroop and finger tapping tasks. In the present study, we did not see a significant effect on 4CRT. However the findings of Bingham et al. (Bingham, Hopkins et al. 2003) are convincing in that diazoxide and glibenclamide were shown to have the opposite effect on 4CRT performance during hypoglycaemia. Overall, therefore, it is likely that K_{ATP} channel openers may reduce psychomotor speed during hypoglycaemia, and possibly lower the threshold for cognitive deterioration. The question is whether this change has clinical significance. During hypoglycaemia all neuronal cells will be exposed to low glucose and are likely to adapt to this physiological stressor. This is consistent with our findings of a greater counterregulatory hormone response and deterioration in cognitive performance (as well as a tendency to lower thresholds for change) following diazoxide in long-standing type 1 diabetes. In agreement with our findings, long-term avoidance of hypoglycaemia in T1D (Fanelli, Epifano et al. 1993) as well as surgical removal of insulinoma to cure chronic hypoglycaemia

in non-diabetic subjects (Mitrakou, Fanelli et al. 1993) results in lower thresholds for cognitive, symptomatic and hormonal counterregulatory responses to hypoglycaemia while experimentally induced chronic hypoglycaemia does the converse (Boyle, Kempers et al. 1995). In the context of the present study therefore oral diazoxide appears to have widespread effects on brain function leading to an overall reversal of hypoglycaemia-induced adaptations in brain glucose sensing and psychomotor performance.

Finally, in this study we make the interesting observation that the presence of the E23K polymorphism predicted to a large extent whether an individual would respond to diazoxide during hypoglycaemia. The exact prevalence of the E23K polymorphism has previously never been studied in the T1D population. This polymorphism was first identified in a meta-analysis study in French Caucasians, which showed that the single nucleotide polymorphisms (SNPs) at codon 23 (E23K,rs5219) in Kir 6.2 (which is encoded by the *KCNJ11*, gene) was associated with Type 2 Diabetes (Hani, Boutin et al. 1998) and also with better response to sulphonyureas (Javorsky, Klimcakova et al. 2012). The K23 variant of the K_{ATP} channel results in a 60% increase in the likelihood of the K_{ATP} channel being open in the resting phase compared to the wild type E23 form, and although this variant is in the pore-forming Kir6.2 channel, it demonstrates strong allelic association with a coding variant (A1369S) in the neighbouring SUR1 gene thus predicting response to sulfonylureas (Florez, Burt et al. 2004, Sesti, Laratta et al. 2006). In our small cohort of 12 participants with well-established T1D, we found 58% carried the K23 variant. This is comparable with the prevalence of 51% (41% hetero- and 10% homozygote) for the E23k polymorphism reported in participants with pre-diabetes in the Diabetes Prevention Program (Florez, Burt et al. 2004) and with 63% and 59% respectively of type 2 diabetic subjects in the UKPDS and normoglycaemic control subjects (Gloyn, Hashim et al. 2001). Although the small size of our study cohort limits the conclusions we can reliably draw from this analysis, our data suggest that the E23K polymorphism may identify individuals

with T1D both at increased risk of developing IAH and/or requiring a greater dose of diazoxide to amplify the counterregulatory response to hypoglycaemia. Future studies will be required to address this hypothesis.

In summary, we have shown for the first time in human subjects, that the K_{ATP} channels are integral to hypoglycaemia detection and in the generation of an adequate CRR to acute hypoglycaemia. We report that, the K_{ATP} channel opener diazoxide, when given orally prior to a hypoglycemic stimulus to subjects with long-standing T1D and IAH, results in a 37-44% increase in the magnitude of the catecholaminergic counterregulatory hormonal response. Moreover, our data suggest more widespread central actions of diazoxide on neuronal populations involved in psychomotor responses and symptom generation. Finally, we have made the novel observation that the E23K polymorphism in the Kir6.2 subunit of the K_{ATP} channel predicts response to diazoxide therapy during hypoglycaemia in T1D.

CHAPTER 4

So our first approach to reducing hypoglycaemia in patients with T1D, was through modulation of the glucose sensing apparatus, but a further likely target which has recently come to light is that of glucose variability (GV). GV has been shown to be important in short term outcomes, and has been shown to predict periods of marked glucose excursions particularly hypoglycaemia {Cox, 1994 #24946} {Kilpatrick, 2007 #24947}. Particularly the combination of elevated HbA1c and increased GV has been shown to be associated with increased frequency and severity of hypoglycaemia, with increased GV shown to precede episodes of hypoglycaemia (Kovatchev, Cox et al. 2000). A re-analysis of the DCCT cohort, showed that both mean blood glucose (MBG) and GV were able to independently add to HbA1c in predicting risk of hypoglycaemia. It was shown that a mean rise of 1mmol/L in MBG resulted in a 1.05 reduction in SH, whereas a 1 mmol increase in standard deviation (SD), showed a 1.07 increase in risk of hypoglycaemia (Kilpatrick, Rigby et al. 2007).

GV has also been found to have a relationship with the counter-regulatory responses generated during acute insulin induced hypoglycaemia. In a univariate analysis of 28 adolescents with T1D, the coefficient of variation (CV), a measure of GV, was shown to be negatively correlated with glucagon concentration during insulin induced hypoglycaemia, however in the multivariate analysis, only Continuous overall net glycaemic action (CONGA), another measure of GV, was shown to be negatively correlated with a glucagon, which was independent of other factors such as HbA1c and duration of diabetes. However, other counter-regulatory hormones were not affected (Alghothani and Dungan 2011). Low Blood Glucose index (LBGI), an index which is designed to be sensitive to hypoglycaemia alone, also showed that the higher the LBGI (suggestive of increased burden of hypoglycaemia), the lower the magnitude of epinephrine responses during induced

hypoglycaemia ($p=0.009$). GV as assessed through measurement of SD of blood glucose (from 70 SMBG readings over 4 weeks) in a separate cohort of T1D patients at baseline however was also shown to be a highly significant predictor of hypoglycaemic unawareness ($p=0.001$)(Bragd, Adamson et al. 2008)

Apart from increased GV possibly resulting in a defective counter-regulation and symptom generation during hypoglycaemia, GV has also been shown to induce proinflammatory, prothrombotic pathways and induce oxidative stress. Insulin induced hypoglycaemia clamp studies have shown production of adhesion molecules, intracellular adhesion molecules (ICAM), vascular cell adhesion molecules (VCAM), E selectin, P-selectin, cytokines, interleukin-6, plasminogen activator inhibitor-1 (PAI-1) and vascular endothelial growth factors (VEGF) factors

Therefore our 2nd study (STUDY 2) seeks to determine if an adjunct therapeutic agent could be utilised to reduce glycaemic variability and hence reduce the frequency of hypoglycaemia thereby improving symptom generation and awareness in those with T1D. Prior to discussion of STUDY 2, following is a discussion on GV, what it is, its importance and how it can be reliably measured?

Glucose variability

Definition

Glucose variability is defined by the swings in blood glucose. HbA1c has been the gold standard for assessment of glycaemic load, but it includes glucose excursions from hypoglycaemia to euglycaemia to fasting and post-prandial hyperglycaemia. HbA1c therefore only marginally represents the full dynamics of glucose regulation, and clearly other parameters are needed to assess the role of glycaemic variability in health and disease.

Why does it occur in Type 1 Diabetes?

Glucose variations seen in those with T1D are usually a result of the interplay between diminished glucose counter-regulation during hypoglycaemia and the shortcomings of current available insulin therapy that lead to hyperglycaemia. The result is that glucose homeostasis is profoundly disturbed and the individual with T1D may experience profound shifts in prevailing glucose levels throughout the day. This occurs even in the context of optimal glycaemic control in well-motivated patients, and can be a source of significant morbidity, with patients having to regularly use supplemental insulin and glucose in order to reduce these fluxes. The problem is exaggerated in less motivated patients with recurrent insulin omission leading to significant glucose fluxes, with significant rises in glucose, prompting large correction doses, often with resultant hypoglycaemia.

Measuring Glucose variability

There is currently no gold standard measure of glucose variability and most groups actively researching in this field use composite measures that provide an index of both the extent (essentially standard deviation from the mean) and duration of glucose exposure outside of the normal range to gauge the total burden experienced by any one individual.

So far we see that the majority of studies have used the DCCT cohort (using 7 point SMBG readings for calculation of GV) and the remaining studies in CGM have only used small timescales of monitoring in order to establish relationship between GV and long term outcomes. Clearly there is a need for establishing the best way of measuring GV- possibly through longer periods of monitoring using CGM.

Most measures of blood glucose variability are primarily dependant on hyperglycaemic blood glucose excursions and are generally insensitive to hypoglycaemia due to the asymmetry of

the blood glucose scale. Since the varying measures have inherent properties depending on the nature of the problem, different measures cannot be used interchangeably. Although measures of GV are closely correlated in non-diabetic subjects (correlation coefficients >0.92), this has not been the case in those with T1D (Cameron, Donath et al. 2010). The consensus is that measures cannot be clumped together, and rather should be chosen on the merits of the index.

A reanalysis of the ADAG data (Kuenen, Borg et al. 2011), showed that there is a significant relationship between mean glucose and HbA1c ($r=0.876$), which is more pronounced in the higher HbA1c levels. Kuenen et al (Kuenen, Borg et al. 2011) showed that both SD and CONGA had a significant relationship with this mean-HbA1c relationship. However once again they also showed that this relationship is poor around the optimal HbA1c range. Therefore it may be more pertinent to measure glucose variability in well controlled patients (optimal HbA1c) as in this group, it would be difficult to predict GV without some form of CGM.

GV has 2 primary components; the vertical and horizontal. The vertical component relates to intra-day variability, corresponding to the within day fluctuations (e.g. SD, MAGE, M value, CONGA, ADRR) whereas the horizontal component relates to day to day glucose fluctuations (MODD).

Below is a list of the glucose variability indices that will be measured in STUDY 3.

Measure: This is a within day variability index.

Advantages: This is easy to calculate

Disadvantages: Does not consider amplitude or frequency of fluxes.

Standard Deviation

Measure: Measure of dispersion rather than of true glucose variability. It shows a linear relationship to mean glucose with an r of 0.56. (Rodbard 2011) Hirsch suggests that in T1D, SD should be no more than mean/2 (and mean/3 in T2D).

Advantages: ease of measurement. SD shows the least change at all measurement intervals. SD has been shown to be higher in T1D with microvascular complications, despite no differences in average glucose levels (HbA1c) (EASD abstract 2012). It can be a tool used in clinical practice as it can be calculated from seven point glucose curves. However even a seven point curve may miss significant excursions, making this less reliable measurement. It (within day SD) has however been shown in an analysis of the DCCT cohort to be an independent predictor for time to first severe hypoglycaemic event (Kilpatrick, Rigby et al. 2007).

Disadvantages: No weighting for minor or major variations.

Glucose profile data has often non-Gaussian distribution, and therefore has a potential for widely different glucose curves to have the identical numerical value of SD. As a result of the hypoglycaemia range being narrower than the hyperglycaemia range, it is much skewed, and therefore, SD would be very influenced by hyperglycaemia excursions and would not be sensitive to hypoglycaemia.

J index

Measure: $0.324 (\text{MBG} + \text{SD})^2$ the relationship between SD and the absolute value of the arithmetic mean glycaemia or J-index. Ideal control 10-20. Good control 20-30. Poor control >40

Advantages: It removes the need for glycaemic reference points. Measures both mean level and glycaemic variability.

Disadvantages: However when first designed, the authors warned that it is invalid for glucose readings less than 1.67 or 3 or more consecutive readings less than 2.78mmol/L. So it is important when reporting this measure, that authors give info about number of hypo and hyperglycaemic episodes (Wojcicki 1995).

Mean absolute glucose

Measure: Summation of all absolute changes in glucose divided by the time over which measurements were taken. (Hermanides, Vriesendorp et al. 2010)

Advantages: 2 excursions of same extent but of different duration have different values. Strong correlations were found between MAG and SD, MAGE, and CONGA. (p<0.001)(Kohnert, Heinke et al. 2013)

Disadvantages: Poor correlation between MAG and mean glucose $r=0.246/0.378$ respectively. No real advantage over other measures of GV.

Mean Amplitude of Glycaemic Excursions (MAGE)

Measure: It was developed using hourly blood glucose sampling over 48 hours. The arithmetic mean of the glycaemic excursions (amplitudes) greater than a pre-specified threshold size (usually 1 SD) for a period of study is the value of MAGE (Service, Molnar et al. 1970)

Advantages: MAGE is not dependent on the mean glucose value, and only looks at major excursions and excludes minor ones (<1SD)

Disadvantages: The outcome differs depending on whether ascending or descending limbs are used for the calculation of MAGE, with as much as 7% difference. Only excursions greater than 1SD are included, and therefore, exclusion of anything else, may exclude clinically relevant excursions. Due to the asymmetry of the glucose range, MAGE tends to have a bias towards hyperglycaemic peaks and is largely insensitive to hypoglycaemic nadirs. There are still no normal ranges for people with diabetes. MAGE becomes very unreliable if there glucose measurements are more than 1h apart (due to connection failure)(Baghurst, Rodbard et al. 2010).

Mean of the daily differences (MODD)

Measure: This is a measure of inter-day glycaemic variability, and measures the mean of the absolute differences between glucose values at the same time of the day during different days of measurement.

M value of Schlichtkrull: 1965

Measure: Mean of the logarithmic transformation of the deviation from a reference value of 6 blood sugar measurements taken over a 24 h period plus an amplitude correction factor.(ACF) ($ACF = \frac{\text{max} - \text{min blood sugar for a 24 hour period}}{20}$). The ideal glucose value of 6.6mmol/L was first assigned based on 20 patients with T1D(Schlichtkrull, Munck et al. 1965) However, the ideal glucose value can now be allocated by the individual investigator, making comparison of studies challenging. M value is 0 in healthy controls, rising with increasing variability or poorer glycaemic control, so it is difficult to distinguish between high variability and high mean glucose. Hypoglycaemia has a bigger impact on the M value than hyperglycaemia.

Advantage: More emphasis on Hypoglycaemia than hyperglycaemia. Hybrid measurement of variability and mean glycaemia. A single M value expresses both mean glucose value and the effect of glucose swings.

Disadvantages: Inappropriate for CGM use.

Continuous overall net glycaemic action (CONGA-n)

Measure: It is a good measure of rapid glucose variability. Designed for CGM readings.

For each glucose point after the first n hours of observations, the difference between current glucose and the glucose n hours previous is calculated. CONGA is expressed as the SD of the differences. Higher CONGA readings indicate greater GV.

Advantage: Designed specifically for CGM. It is not affected by the asymmetry of the blood glucose range. It also does not require identification of peak or nadirs according to arbitrary definitions.

Disadvantages: Full dataset required. The SD of the differences are not normally distributed, and therefore has implications on calculation of the CONGA.

Low Blood Glucose Index and High Blood Glucose Index

Measure: It involves correction of the skewness of glycaemia (narrow hypoglycaemia vs broad hyperglycaemia) through a symmetrisation process around zero.

($\approx 112.5\text{mg/dL}/6.2\text{mmol/L}$) by expanding the hypoglycaemia range and reducing the

hyperglycaemia range (Kovatchev, Cox et al. 1997) The JDRF CGM study showed that CGM hypoglycaemia >30% of the day was highly correlated with LBGI, AUC and each of these increased the probability of SH by 8 fold, but because SH is a rare event, cannot be used as a

predictor. (Fiallo-Scharer, Cheng et al. 2011) However another study showed a history of SH and LBGI calculated from the previous month's SMBG accounted for 40% of variance of SH over the following 6 months. (Kovatchev, Cox et al. 1998) LBGI has been shown to be predictive of SH with a modest sensitivity rate of 58-60% among 100 T1D. (Cox, Gonder-Frederick et al. 2007) LBGI; 4 categories of LBGI have been reported with regard to risk of future hypoglycaemia. Those in the minimal risk experienced no severe hypoglycaemic episodes, however those in the high risk experienced on average of 5 severe episodes over the following 3 months. (Kovatchev, Cox et al. 2003)

Advantages: LBGI has been shown to be a powerful predictor of severe hypoglycaemia. The accepted ranges are $LBGI < 1.1$; Minimal risk, $1.1 < LBGI < 2.5$; Low risk, $2.5 < LBGI < 5$ Moderate risk, > 5 High risk.

High Blood Glucose Index.

Measure: Estimate of the risk and magnitude of hyperglycaemic excursions.

Advantages: HBGI was found to be correlational to the HbA1c. The accepted categories currently are $HBGI < 4.5$ Low risk; $4.5 < HBGI < 9$ Moderate Risk and > 9 High risk

Disadvantages: LBGI and HBGI do not correlate with their opposite blood glucose ranges.

Glycaemic risk assessment diabetes equation (GRADE)

Measure: This was designed for both SMBG as well as CGM data. Each BG value is converted to a GRADE value. The higher the GRADE, higher is the GV. In order to know where the major problems lie, the percentage of time spent in hypoglycaemia ($GRADE < 3.9$), euglycaemia ($3.9 < GRADE < 7.8$) and hyperglycaemia ($GRADE > 7.8$) are also calculated. So percentage gives the reader an estimate of where the majority of the fluxes lie. It is therefore, a measure of risk consequent of GV, rather than a measure of GV.

Advantages: Can be used for CGM. Independent of observer bias and being able to deal with asymmetric profiles.

Average Daily Risk Range

Measure: It was initially used for SMBG readings (at least 3-5 readings/day) for 30 days, but increasingly is also used in CGM profiles. The dataset (39T1D, 31T2D) used for the development of this ADDR, showed that ADDR was correlated to future extreme hypoglycaemia (<2.2) and extreme hyperglycaemia (>22.2) by 0.40 and 0.53 respectively and the correlation between ADDR and mild hypo (<3.9) and mild hyper (>10) were 0.41 and 0.63 (Kovatchev, Otto et al. 2006). Its values have been stratified into three categories; low risk ADDR <20 ; Moderate risk ADDR 20-40; High risk ADDR >40

Advantages: Unlike the other measures, which focus more on the either hypoglycaemia or hyperglycaemia, the ADDR, is designed to be equally sensitive to hypoglycaemia and hyperglycaemia. It has been found to be a good predictor for glucose fluctuations as well as higher frequency and severity of events. It has been shown to be the best predictor for both extremes of the glucose range, with a 6 fold increase and 3.5 fold increase in the likelihood of hypoglycaemia and hyperglycaemia respectively across the risk categories. Risk scores are used for calculation rather than actual blood glucose values, and therefore glucose range is given less weight.

Disadvantages: No formal assessment with CGM.

The above measures were chosen in STUDY 3, because they included methods that relate to SD (Mean, SD, J index, MAG), methods which relate to glucose excursions (MAGE, GRADE) methods relating to day to day variability (MODD-mean of daily differences)) and methods that look at short term variability over hours (CONGA-Continuous overall net

glycaemic action and M value). We also looked at measures which calculated the burden of hypoglycaemia and hyperglycaemia (LBGI and HBGI) and a combination of them both (ADRR).

So far the literature does not suggest a universal measure of GV, making comparison of studies challenging. So currently, measures need to be chosen on their own merit, and according to the particular component of variability that is of interest to the researcher, and answers a relevant question.

Technicalities of measuring GV

It is important to collect data which is able to give reliable and consistent measures of GV. It has been shown that as the interval between observations increase, so does the variability of GV indices (Baghurst, Rodbard et al. 2010). SD appears to be the most hardy of all the measurement intervals, however if the time interval is greater than 2 hours apart, both SD and CONGA4 becomes unreliable, with MAGE exhibiting the highest systematic change (unreliable if readings >1 hr apart). Most of the current CGM devices available on the market, give 5 minute readings, so the above is only a consideration, if there is gap in the CGM trace/incorrect calibration/temporary disconnection of sensor).

Subjects invariably change behaviour when attached to a CGM monitor, and optimal duration of measurement is still under debate. Conclusions regarding long term complications have been made on monitoring periods as short as 72 hours in several studies (Sartore, Chilelli et al. 2013). This assumes this period of monitoring is reflective of their usual glycaemic control. However, unless there is some standardisation of both diet and exercise, any changes from the norm of these, can result in abnormal glucose variations being captured in the period

of monitoring and influence measures of GV significantly. A recent study in a paediatric cohort (20pts) compared varying number of days (2-30) days of CGM data with the gold standard 90 day measurement of GV. There were significant gaps in the CGM trace (mean 446.2 ± 239 hours per 90day period, so only SD and CV could be used for reporting. Naturally as CGM duration increased, there was a reduction in %error from the 90 day value, but reached a median value of <10% error at 12 days. However in a study in adults, 6 days was thought to be sufficient to assess GV using MAGE. This shorter time period maybe because of increased stability of the glucose profile in adults(bugler 2008).

Associations between GV indices

A study was done in Japan (20 T1D, 68 T2D), with CGM looking at the association between different measures of GV{Saisho, 2015 #24945}. Strong correlations were found between SD and MAGE, CONGA-1, MODD and M value (all $r > 0.8$, $p < 0.05$). Mean glucose was correlated with J index and M value ($r > 0.8$, $p < 0.05$). SD was more strongly correlated with hypoglycaemia than was mean glucose, with a combination of Mean and SD increasing the predictive value of hypoglycaemia.

Is it important?

There is much debate as to whether glycaemic variations in those with T1D are clinically pertinent. It was shown recently, that despite patients having an identical HbA1c in the conventional and intensive arms of the DCCT, the former group had an accelerated risk of progression of retinopathy over time. This difference was initially attributed to markedly higher glucose variability in those on conventional treatment compared to those on intensive insulin therapy.(Brownlee and Hirsch 2006). Other factors which have thought to be contributory include fasting glucose levels, post-prandial hyperglycaemia(Ceriello 2005) and

longer term variability (HbA1c variability-HR 2.26 for every 1% increase in A1c SD, $p<0.0001$)(Kilpatrick, Rigby et al. 2008). Indeed the ADAG study, an international multicentre study, showed there is a wide range of average blood glucose readings associated with a particular HbA1c(Nathan, Kuenen et al. 2008). This was confirmed by the Juvenile Diabetes Continuous Glucose Monitor Study(Wilson, Xing et al. 2011) which once again showed wide mean sensor glucose concentrations for a calculated HbA1c.

However, debate as to the significance of these fluctuations continued as reanalysis by the DCCT/EDIC cohort showed that after adjusting for retinopathy at baseline and adjusting for updated HbA1c as a time dependant covariate, it was found that HbA1c indeed accounted for 96.2% of all the treatment effect. (Lachin, Genuth et al. 2008).

A further DCCT re-analysis by Kilpatrick et al, (Kilpatrick, Rigby et al. 2008) showed using multivariate Cox regression analysis that HbA1c variability itself, adds to the risk score of development of microvascular complications. They found that for every 1% increase in HbA1c standard deviation, the risk of retinopathy increases by 54% (95%CI 1.07-2.32 $p=0.02$) and for nephropathy 42% (95% CI 1.00-2.00 $p=0.05$) suggesting that more long term variability is more harmful than short term variability. This relationship continued to exist on follow up, when the EDIC data (after 4 years follow up), showed that both mean A1c and mean glucose were highly predictive of retinopathy ($p<0.001$), with mean A1c being also predictive of nephropathy. However as hinted by the original DCCT, EDIC also showed that GV (assessed with standard deviation (SD) and mean amplitude of glycaemic excursions (MAGE) did not predict the development of diabetes related microvascular complications(Kilpatrick, Rigby et al. 2009).

The underlying idea for considering glucose variability is that it, similar to hyperglycaemia, may be associated with mortality by increasing oxidative stress, neuronal damage,

mitochondrial damage, and coagulation activity (Brownlee 2005, Monnier, Mas et al. 2006, Egi and Bellomo 2009). It is well established that hyperglycaemia leads to end organ damage, through accumulation of non-metabolizable sugars. This is through several mechanisms such as polyol pathway particularly used in those tissues which are non-insulin requiring (nerves, lens), whereby glucose enters, and is converted to non-metabolised sugars such as fructose and sorbitol, causing both osmotic changes and depletion of key cellular components such as NADPH. Furthermore there is also glycation of proteins such as collagen resulting in tissue and organ damage, formation of advanced glycosylated end products (AGEs), which can be a trigger to multiple cytokine pathways, which eventually lead to activation of protein kinase C, mitogen activated protein kinase (MAPK) and nuclear factor kappa B, which ultimately lead to oxidative stress.

However, rapid fluctuations of blood glucose is thought to produce even more oxidative stress and be more detrimental to vascular endothelium than sustained hyperglycaemia (Monnier, Mas et al. 2006, Giacco and Brownlee 2010). More recently, a number of studies in rodent models, albeit in different organ systems, have suggested that this effect is exacerbated in those animals exposed to hypoglycaemia. For instance, glucose reperfusion following a period of hypoglycaemia was shown to stimulate superoxide production and induce neuronal death (Quagliaro, Piconi et al. 2003). Interestingly, the extent of superoxide production was found to be directly proportional to the final glucose concentrations achieved during reperfusion.

Preclinical studies

Studies in different cell lines have shown that mesangial and tubulo-interstitial cells when exposed to intermittent high glucose concentrations showed an increase in matrix production whereas in pancreatic β cells(Shi, Ren et al. 2011) and endothelial cells(Risso, Mercuri et al. 2001), increased apoptosis was noted. These changes were markedly greater than placing the cells in high glucose continuously(Ceriello and Ihnat 2010).

In vitro and vivo studies(El-Osta, Brasacchio et al. 2008) show that intermittent spikes of hyperglycaemia (6-16 hours) can cause epigenetic changes in the promotor region of the NF κ B of aortic endothelial cells and consequently cause increase in p65 gene expression. This increases expression of the pro-inflammatory proteins monocyte chemoattractant protein-1, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1(ICAM-1), interleukin-6 and inducible nitric oxide synthetase. The duration of these spikes are typically not thought to affect average HbA1c and so would not be accounted for by the crude measurement.

In vivo and rodent models have shown that both hyperglycaemia and glucose fluxes are damaging to cells through the process of oxidative stress. It has been proposed that during these short term swings in glucose, cells are unable to up-regulate their anti-oxidant potential sufficiently to prevent development of diabetes related complications. Particularly, it has been shown that there is an exaggeration of cell damage during hyperglycaemia following hypoglycaemia (similar to reperfusion injury), than there is, during prolonged hyperglycaemia. This has been shown to occur predominantly because of the reperfusion injury caused by increased NADPH oxidase which is exaggerated if blood sugars are left to rise into the hyperglycaemic range.(Cryer 2007).

Effect of GV on the vasculature

Although the in-vitro and vivo studies convincingly show that glycaemic variability causes much oxidative stress, the results in patients with T1 have been less convincing. Wentholt et al, showed that glucose fluctuations (MODD, MAGE, CONGA-n) assessed during CGM (over 3 days), bore no relationship with oxidative stress (measured by urinary excretion of 15(S)-8-iso-PGF_{2α}), although patients with T1 had higher levels of oxidative stress than control subjects(Wentholt, Kulik et al. 2008).

This lack of relationship of GV and oxidative stress seen in those with T1, may be explained by the anti-oxidant property of insulin (Monnier, Colette et al. 2010). Insulin when added to vascular smooth muscle cells was shown to inhibit activation of NF-κB, which is known to be a pro-inflammatory transcription factor activated by glucose. This was seen when there was increased activation of oxidative stress in non-insulin treated T2D patients compared with T2 patients on insulin and T1D patients.

However, Ceriello et al, have performed numerous clamp studies on controls and patients with T1D, which has convincingly shown that fluxes in glucose (particularly hyperglycaemia following hypoglycaemia) can be deleterious to several parameters affecting the endothelium, (significant worsening of FMD –marker of endothelial dysfunction, and sICAM-1, IL6 – markers of inflammation(Ceriello, Novials et al. 2012) (significant increases in vWF, thrombin-antithrombin III complexes, P selectin, plasminogen activator inhibitor 1, nitrotyrosine and 8 iso-prostaglandin F_{2α} – markers of thrombosis activation and oxidative stress)(Ceriello, Novials et al. 2014). The increase in these factors however, were partially suppressed when vitamin C was co-infused. Thus, in the post-hypoglycaemic state, reperfusion of cells with super physiological levels of glucose, a situation that is very commonly seen in clinical practice, may evoke a more marked oxidative stress response.

Taken together, these studies suggest that increased glucose variability with recurrent exposure to hypoglycaemia may induce a pro-inflammatory state that exacerbates endothelial dysfunction in T1D over and above that seen with chronic hyperglycaemia alone.

The relationship appears stronger between GV and cardiovascular outcomes. A study in those with T1D examined a group using RT-CGM and blinded CGM, showed that overall standard deviation (all glucose values) and standard deviation of the mean glucose of each day, showed that these measures of GV (over 3-5 days) were significantly correlated with coronary artery calcification (CAC). This relationship was only present in men. It is known that healthy men have a higher degree of oxidative stress compared to pre-menopausal women. The combination of this background level and increased GV, may then explain this association which was only found in men(Ide, Tsutsui et al. 2002). No association was found between HbA1c and CAC(Snell-Bergeon, Roman et al. 2010). Similarly a retrospective analysis(Kilpatrick, Rigby et al. 2008) of the DCCT/EDIC dataset showed a stronger association between 7 point SMBG (mean blood glucose calculated) and cardiovascular outcomes than with HbA1c.

However with the increased use of continuous glucose monitoring (CGM) systems, studies can now utilise this technology prospectively to investigate the relationship between short term glucose variability and adverse outcomes, and overcome some of the difficulties with SMBG.

Effect of GV on Diabetes related complications

Studies have shown that those with T1D have much higher levels of GV and 24-hour urinary urine 8-iso Prostaglandin F2 alpha (PGF2),a marker of oxidative stress, compared to those with T2D and non-diabetic subjects(Wentholt, Kulik et al. 2008) and those with

complications have been shown to have a further worsening in their GV(Soupal, Skrha et al. 2014).

Both the DCCT (1993) and the UKPDS (Stratton, Adler et al. 2000) have shown good correlation between protein glycosylation and risk of diabetes related complications. It has therefore been seen as the main driving force for development of complications. Although there is inconclusive evidence as to whether GV is related to long term outcomes, particularly after the reanalysis of the DCCT data which showed GV was not related to microvascular complication(Kilpatrick, Rigby et al. 2009).

A recent systematic review(Nalysnyk, Hernandez-Medina et al. 2010) examined the link between GV and diabetes related complications. Studies in T1D (8 studies) showed that GV had little impact on development of diabetes complications. Only 2/8 showed any association between GV and microvascular complications (not macro). This showed that the majority of studies regarding microvascular complications, were from the DCCT/EDIC database (5 out of 8 studies). The main limitation using this database was the form of assessment of GV. GV in the DCCT cohort was measured by 7 point SMBG- which were pre and post-prandial laboratory measurements over the wakening period on one day, every 3 months throughout the trial(Rohlfing, Wiedmeyer et al. 2002). This did not take into account nocturnal readings, and therefore could have missed important glucose fluctuations. Currently there is only one study looking at CGM data and microvascular complications. This study combined both patients with T1D and T2D, probably to increase statistical power, and once again showed no significant relationship in multivariate analysis between GV (measured by SD, MAGE, and CONGA-2) and diabetic retinopathy. Combining T1 and T2 may not be very informative, as studies looking at solely at patients with T2D, suggest an opposite effect to those in T1D, with GV (particularly in fasting plasma glucose) having a greater impact on microvascular complications in those with T2D(Smith-Palmer, Brandle et al. 2014). GV was shown to

increase urinary markers of oxidative stress in patients with T2D (Monnier, Mas et al. 2006), and it is thought that the rise in reactive oxygen species (ROS) contributes to the development of complications. Monnier et al (Monnier, Colette et al. 2012), proposed that GV may have a differing effect in T1D and T2D, because exogenous insulin has been shown to have an inhibitory effect on the activation of oxidative stress pathways, and thereby may potentially blunt the effect of glucose fluxes on the vasculature.

A recent study utilising CGM (5 day) in patients with T1D, showed that LBGI- an index of GV reflecting hypoglycaemic stress was associated with reduced low frequency (LF) and high frequency (HF) heart rate variability, which can indicate impaired autonomic function. HbA1c did not however correlate to these measures (Jaiswal, McKeon et al. 2014). This was a stark contrast to the findings from the reanalysis of the DCCT cohort, which utilised 7 SMBG readings, and found no correlation between GV and autonomic function.

Effect of GV/recurrent hypoglycaemia on the Hypothalamo-pituitary-adrenal axis (HPA)

Hypoglycaemia presents a profound stimulation to the HPA axis and stimulates a rise in peripheral glucocorticoid, while repeated hypoglycaemia may eventually lead to a dysregulation of this response. GV through its strong association to recurrent hypoglycaemia could potentially also cause dysregulation of the HPA system. This represents a form of ‘stress habituation’, which could potentially lead to blunting of cortisol diurnal variation (Heim, Ehlert et al. 2000). In the Whitehall II study, blunting of the diurnal cortisol curve was linked to increased risk of all-cause mortality (Kumari, Shipley et al. 2011). Diurnal cortisol decline has also been related to increased coronary calcification in the CARDIA study. (Matthews, Schwartz et al. 2006). Thus, we hypothesized that increased glucose variability

and repeated hypoglycaemia may, independently from chronic hyperglycaemia, contribute to cardiovascular disease (CVD) mortality in T1DM through dysregulation of the HPA axis,.

If there is indeed blunting of the cortisol decline, this would be in parallel to an increase in cortisol AUC. It is well recognised that pathological hypercortisolaemia seen for instance in conditions like Cushing's syndrome can result in glucose intolerance, insulin resistance and other cardiovascular risk factors such as hypertension and hyperlipidaemia. However, even within the physiological range, variations of cortisol may also contribute to the development of these risk factors. Hypercortisolism, hypocortisolism, and a blunted diurnal cortisol slope have all been associated with adverse measures of mental and physical health. (Champaneri, Wand et al. 2010). Kirschbaum and colleagues have suggested that the HPA axis can be useful marker for the consequences likely to occur as a result of prolonged exposure to stress(Kirschbaum and Hellhammer 1999).

Role of Glucocorticoids (GC)

GC has regulatory functions involving metabolism whereby, it stimulates gluconeogenesis in the liver(Jitrapakdee 2012) and controls deposition and breakdown of lipids. Cortisol (the main GC present in humans) has been implicated in the rise in insulin resistance seen in the early hours of the morning, associated with a peak in its level. It regulates several immune and inflammatory responses (Munck and Naray-Fejes-Toth 1992). Cortisol has a range of effects including blood pressure elevation and modulation of cell growth. It has a multitude of central effects such as changes in mood and behaviour, it affects food intake, body temperature and nociception. (Chrousos and Kino 2007) and plays a role in memory formation. (Trollope, Gutierrez-Mecinas et al. 2012) Cortisol also has a sleep regulatory function, with a study(Born, Spath-Schwalbe et al. 1989) suggesting that rises in cortisol

decreased the time spent in Rapid Eye Movement (REM) sleep but increased the time spent in Slow Wave Sleep (SWS).

Normal cortisol patterns

There is a distinct pattern of cortisol secretion resulting in a circadian rhythm, reaching a nadir around 3am to reach a peak around 9am, and then falling throughout the day. (Krieger, Allen et al. 1971). In humans, this prominent circadian rhythm (Stratakis and Chrousos 1995) is seen in both plasma cortisol and corticosterone concentration with an approximate 13:1 ratio maintained throughout the 24 hour day. This circadian rhythm (also involving ACTH) also reflects an underlying ultradian rhythm(Veldhuis, Iranmanesh et al. 1989) (Henley, Leendertz et al. 2009). The ultradian pattern was discovered in one of the earliest studies(Veldhuis, Iranmanesh et al. 1989) whereby blood samples were taken at 10 minute intervals over a 24 hour period in 6 healthy men and showed randomly occurring cortisol bursts at a mean frequency of 19 ± 0.82 events per day (interpulse interval 77 ± 4 mins). The burst frequency and amplitude varied 2.2 and 6.6 fold respectively over the 24 hour period. Burst amplitude rather than the burst frequency is thought to influence the circadian rhythm(Liu, Kazer et al. 1987), as these rapid fluctuations have been shown to affect the responsiveness of the glucocorticoid responsive genes(Lightman, Wiles et al. 2008).

The levels of GC are higher during the activity period in most mammals with the highest levels in the 2nd part of the night with peak levels at the start of the activity period (morning for humans). Following this, there is a decline in cortisol levels with the lowest levels in the first part of the night. Many investigators have suggested that the “crispness” of the cortisol response is of vital importance i.e. a good sharp rise in the morning, followed by an elegant

decline, for good health. This pattern has been repeatedly shown to be associated with a healthy population.

Overlying this basal rhythm, the HPA axis is also responsive to several stressors including hypoglycaemia, and reaches a peak at 15-30 mins after HPA activation, returning back to basal levels within 60-90 minutes (de Kloet, Joels et al. 2005), and plays a key role in the body's adaptive responses to various types of stress.

Components of the normal diurnal pattern

Morning rise- Cortisol Awakening Response (CAR)

In addition to the normal diurnal pattern of cortisol secretion, there is an additional burst of cortisol (rise of 50-100%) release within the first 30-60 minutes following waking, which is known as the cortisol awakening response (CAR). Wakening is a potent stimulus of the HPA axis, and the rise in cortisol seen is thought to be a marker of stress reactivity/adrenocortical activity. In particular the increased level of glucocorticoids in the morning prepare the body for the impending day, and by increasing energy levels, are able to enable foraging behaviours.

It has been shown to be more in tune with our natural sleep/wake cycle and superimposes the underlying increase in cortisol seen in the early morning hours as a result of the activation of the HPA axis. (Wilhelm, Born et al. 2007). It usually peaks at about 30 mins after waking and can remain elevated for up to 60mins. CAR is thought to be crucial in attainment of full alertness through possible activation of the prefrontal cortex.

It has recently come to light that regulation of CAR is under neural control. The SCN appears to provide direct neural stimulation of the adrenal zona fasciculata (innervated by sympathetic neurons) which increases its sensitivity to ACTH from the pituitary, and

enhances cortisol secretion in the immediate waking period(Clown, Hucklebridge et al. 2010).

Furthermore, hippocampal volume has been positively associated with magnitude of CAR, with those with either lesions or those with retrograde amnesia, showing no CAR response(Fries, Dettenborn et al. 2009). However, the role of the hippocampus on the HPA axis is still debated. The high concentration of gluco- and mineralocorticoid receptors on the hippocampus suggests a crucial role. Some studies suggest a role in the negative feedback regulation of the HPA axis, whereas others provide evidence for a stimulatory role on the axis(Fries, Dettenborn et al. 2009).

If done with strict adherence to the sampling protocol (measurements done within the first 30-60mins after waking), then CAR has been shown to have a relatively good intra-individual stability across days and weeks (Pruessner, Wolf et al. 1997, Wust, Wolf et al. 2000). However Kudielka et al, showed that flattening of the CAR was in certain individuals associated with non-compliance when they were electronically monitored. (Kudielka, Broderick et al. 2003). It has also been estimated that about 15% of individuals will not have a cortisol rise.(Dockray, Bhattacharyya et al. 2008). The CAR is often assessed by self-collection of salivary collections usually in a domestic setting. Several samples need to be obtained within the first 60 minutes after waking. The mode of waking (with or without an alarm clock) has not been shown to impact on the CAR(Wust, Wolf et al. 2000).

Literature suggests that there is a certain heritability of morning cortisol levels (Kupper, de Geus et al. 2005, Steptoe, van Jaarsveld et al. 2009). A recent study has indicated that a novel GLP-1 variant (rs1042044) is associated with HPA axis function, with children homozygous for the phenylalanine allele showing significantly higher salivary cortisol levels cf leucine homozygosity(Sheikh, Dougherty et al. 2010).

CAR has been shown to be independent of time of awakening, total time slept and sleep quality(Wust, Wolf et al. 2000). In four independent studies involving 509 adult subjects, it has been shown that neither age, use of oral contraceptive pills, habitual smoking, wakening time (when it was in the normal range), total time slept had any impact on the CAR(Wust, Wolf et al. 2000). However if subjects got up out with of the normal range, (between 4 and 5am or between 11.30 and 2.30), then it has been shown to cause either an enhanced or diminished CAR response respectively(Wust, Wolf et al. 2000). The magnitude of rise has been shown to be influenced by gender (females have a larger CAR)(Wust, Wolf et al. 2000) and age (increasing age associated with lower CAR) pain-(attenuated elevation-lower CAR) (Geiss, Varadi et al. 1997) and chronic stress-(lower CAR). Disturbances are thought to relate to changes in the HPA axis, and may serve as an indicator of allostatic load (cumulative minor stresses over a period of time).

The significance of CAR is still highly debated, despite many published studies on the function of this rapid rise in cortisol after wakening. However, there are a few findings that have been repeated in different studies. It appears that CAR is only mounted when the individual gets out of bed and starts on their daily routine. There appears to be no CAR response at other wakening periods (waking up in the middle of night to go back to sleep, after a short nap –up to 2 hours). CAR appears to be increased in subjects with increased anticipated demands on their time after wakening, with increased CAR on work days compared to non-work days. It is also more influenced by upcoming demands on the day, rather than chronic stress over a period of time. These observations therefore suggest that CAR is highly associated with anticipation of upcoming demands, rather than allostatic load.

The association between physical health outcomes and CAR have been inconsistent, with difficulties in salivary sampling and the inability to control external factors such as upcoming stress making analysis challenging. CAR has also recently been found to be associated with

executive functioning. A recent paper in the healthy old, showed that there was a strong relationship between CAR profiles and measures of Trail making test performance (which is a frontal lobe dependant domain). The study in 50 participants showed that those who had better executive functioning had an earlier peak and greater magnitude of the CAR (Evans, Hucklebridge et al. 2012). A study in Netherlands showed that an absent CAR was associated with increased cardiovascular event rate (Vreeburg, Kruijtzter et al. 2009). To look at in greater detail, various measurements can be calculated

Table 5 CAR has been measured in a variety of ways in published studies

| Measure of CAR | | Description |
|--|---|---|
| AUC(i)(Pruessner, Kirschbaum et al. 2003) | Area under the curve with respect to the increase | Measure of the dynamic of the CAR |
| AUC (g)(Pruessner, Kirschbaum et al. 2003) | Area under the curve with respect to the ground. | Total cortisol in the 1 st hour of wakening |
| Mean increase (Wust, Wolf et al. 2000) | | Subtracting the baseline from the mean of the other values in the first hour $(F0.5 + F0.75)/2 - F0$ |

Slope

In healthy individuals, HPA axis has a diurnal rhythm with peak cortisol levels usually after 30 minutes on wakening, and then declining cortisol levels over the day, with the lowest level around 3am. A pronounced decline over the day is evident in many studies in healthy individuals, whereas a more gradual decline, may indicate continued HPA over-stimulation. A flatter slope can represent a long term response to chronic stress. This can be seen in non-pathological high stress situations seen in those with marital problems, unemployed

individuals, women undergoing separation anxiety. (Grossi, Perski et al. 2001, Powell, Lovallo et al. 2002, Barnett, Steptoe et al. 2005) This pattern has also been seen in many different disease cohorts (poor maternal relations, post-traumatic stress disorder, poor prognosis in breast cancer patients, poor declarative memory and in those with increased perceived stress (Evans, Fredhoi et al. 2011). The Whitehall II study was the first large prospective cohort study (n=4047) to show flatter slopes were associated with increased all-cause mortality (1SD reduction in slope HR 1.30 (1.09-1.55), and this was mostly attributed to cardiovascular deaths (HR 1.87 (1.32-2.64) (Kumari, Shipley et al. 2011). A flattened diurnal rhythm has also been shown to be linked with the metabolic syndrome (Rosemond, Dalman et al. 1998).

Often the slope is calculated using both a morning sample and the last sample (either before bed or 12 hours after wakening). There is currently no consensus, as to which morning sample to use, as there is a sharp rise in cortisol in the first hour after wakening, and so method of analysis can significantly affect the results. However this may be more of a problem when comparing different studies. So currently, as long as the method is consistent within a study, then comparisons may be made between groups. Investigators have used the wakening sample, the peak CAR (or 45 min sample) or 3 hours following wakening sample for use as the first sample. The last sample has been either the sample before bed or the 12 hour wakening sample. It has been established that CAR (0-45 minute samples-awakening samples) and diurnal (45 mins to 12 hours) patterns are under different regulatory inputs, with poor correlation between these 2 measures(Edwards, Clow et al. 2001).

Since the fastest decline is usually in the first few hours. We calculated the slope in this study using 2 different methods, exploring different sections of the curve.

A. We considered both the 45 minute sample (last waking sample) and the 3 hour sample to calculate the initial downward slope.

B. We also considered the rest of the day samples, and calculated the slope by utilising the 45 minute sample and 12 hours sample.

The last sample can either be 12 hours or prior to sleep. We used 12 hours after awakening. This can be calculated by subtracting the value at last sample from the sample at awakening, and dividing it by the number of hours in between the 2 samples. (Bhattacharyya, Molloy et al. 2008). Other investigators have suggested that the slope can be calculated by subtracting the value at last sample from the peak sample, and once again dividing it by the hours in between the 2 samples.

Area under the curve (AUC) – cortisol

AUC gives a measure of cortisol exposure during the day. As the early rise and slope are under different regulatory controls, investigators suggest that an AUC is calculated for the first 45mins and then for the remaining day. AUC has been shown to be linked with almost all psychosocial measures (such as anxiety, hostility and calmness). Significant hypercortisolaemia (Cushing's and exogenous steroids) and more subtle elevated cortisol has been shown to be linked to cognitive dysfunction. The excess cortisol has been shown to cause widespread effects within the central nervous system. One of the most vulnerable areas is the hippocampus (an area which is important in cognitive function) with its high expression of glucocorticoid receptors (McEwen and Magarinos 1997). It is thought that glucocorticoids inhibit the long term synaptic potentiation (important in memory and learning) (Stranahan, Arumugam et al. 2008). The literature in animals also shows that the baroreflex activity can be altered by circulating corticosterone concentrations (Darlington, Kaship et al. 1989).

There has been a suggestion that increased cortisol may contribute to an increased sympatho-

adrenal tone. This is shown in patients with T2D, where there is evidence of hyper-cortisolaemia where counter-regulation are initiated at normoglycaemic thresholds, indicating an elevated sympathetic neural outflow (Spyer, Hattersley et al. 2000).

Chronic elevation of endogenous glucocorticoids can result in continuous transcription, abnormal mRNA and protein levels. This pattern is seen in many stressful situations such as those with high work stress, lower income and a negative affect. (Steptoe, Kunz-Ebrecht et al. 2003, Ritvanen, Louhevaara et al. 2006). One study showed a significantly ($p=0.035$) higher AUC in adult men who had experienced childhood parental loss (Nicolson 2004). However evidence of a link to concrete physical outcomes is still sparse.

Factors that may affect diurnal patterns

Age

It has been shown that age affects the basal patterns of cortisol release particularly between 2000 and 0130. This is associated with a flattening of the diurnal amplitude, but not the overall pulsatility of the system. (Deuschle, Gotthardt et al. 1997). It is not surprising that more recently, advancing age has also been associated with higher level of cortisol secretion (Wrosch, Miller et al. 2008) with an increased cortisol response to challenge (Otte, Hart et al. 2005). However a recent study showed that these higher levels of cortisol were only detrimental to functional abilities, if there was poor use of control strategies (emotional well-being and good sleeping patterns) (Wrosch, Miller et al. 2009), this finding may account for some of the conflicting data previously about HPA activation and well-being in the older population. The increased exposure to cortisol is likely to lead to accumulation of visceral fat, bone softening and inflammatory dysregulation, and thereby affect a person's ADLs.

Sex

A large non-selected cohort of people (n=1811) in Sweden were examined for differences in cortisol production. Both morning and mean cortisol were significantly elevated in women compared to men(Larsson, Gullberg et al. 2009), whereas males have been shown to have a flatter CAR but a steeper decline in slope(Vreeburg, Kruijtzer et al. 2009).

Smoking

Nicotine binds to the cholinergic receptors in the locus coeruleus and hypothalamus, and activates CRH release and therefore is a potent stimulator of the HPA axis. (Rosecrans and Karin 1998)

Oestrogen

In the context of high oestrogen levels (in those using oestrogen containing contraceptives or in pregnancy women), cortisol released from the adrenal cortex, is more readily bound to the increased levels of cortisol binding globulins, and therefore there is lower concentration of free cortisol which is then measured in the saliva but a higher concentration of total cortisol. It was also found that the peak in cortisol levels was found to be later in the day, suggesting a delay in activation of the HPA axis in the morning. (Meulenberg and Hofman 1990).

Obesity

Studies have shown that those with marked abdominal obesity have a flattened diurnal curve of cortisol secretion.(Putignano, Dubini et al. 2001). Furthermore, the recent Multi-Ethnic Study of Atherosclerosis showed that increasing BMI was positively correlated with an early decline slope (30mins to 2 hours post wakening), despite adjustment for age, race, gender, diabetes status, social status, confounding drugs and smoking status(Champaneri, Xu et al.

2013). In contrast, those with anorexia nervosa have been found to have an overdrive of their HPA axis (Licinio, Wong et al. 1996).

Exercise

The effect of physical activity on basal levels is not known, however there is a clear link of the stimulatory effect of exercise on the cortisol levels, with it being less pronounced in trained as opposed to untrained individuals. (Luger, Deuster et al. 1987, Kirschbaum and Hellhammer 1994) A recent study in Netherlands showed increased activity was associated with increased CAR and a steeper decline in the slope (Vreeburg, Kruijtzer et al. 2009).

Sleep deprivation

It has been shown to cause a pronounced response to breakfast, and is associated with greater AUC with flattening of the slope. This has been shown to be related to higher evening cortisol (Alexander 2003).

Abnormal patterns

A large study- the Whitehall II study looked at 2802 middle aged men and women, and identified two patterns of abnormal diurnal cortisol secretion. They were described as “normative (prevalence 73%)” and “raised (prevalence 27%)”. Raised curves were notably different from the normative curve with a lower cortisol awakening response and a “flatter” pattern of release. This “raised” pattern was associated with increasing age, the male sex, smoking, and stress on the day, slower walking speed and shorter sleep duration. This pattern was also seen to be associated with adverse health behaviours and poor physical functioning (Kumari, Badrick et al. 2010).

Patterns of abnormal regulation of the HPA axis has been shown in many cohort studies as detailed below.

| Healthy cohort | Pattern | Association |
|---|--|--|
| 97 healthy older men (age 65-70) (MacLulich, Deary et al. 2005) | Higher 9am cortisol | Worse age related overall cognitive change, not age related brain atrophy |
| 4047 non-clinical cohort (Whitehall II study) (Kumari, Shipley et al. 2011) | Flatter diurnal slope | Increased risk of all-cause mortality and cardiovascular mortality. |
| Cohort with disease | Pattern | |
| Edinburgh T2 Diabetes study 1,066 men and women with T2D (age 60-75) | Higher fasting cortisol | Associated with estimated cognitive change (this was calculated after adjusting current cognitive function for estimated prior cognitive ability. -lower general cognitive ability -poorer scores on working memory -poorer processing speed. |
| Young Chinese T2D- 90 (age 33±5 years) (Lee, Chan et al. 1999) | Higher basal cortisol | Associated with central obesity |
| Veterans with T2D-154 T2 +52 C(Liu, Bravata et al. 2005) | Higher late night salivary cortisol (LNSC) | Higher LNSC was associated with diabetes and increasing age |
| Veterans with T2D- 216 T2D(Oltmanns, Dodt et al. 2006) | Higher cortisol pre-standardised lunch | Associated with higher SBP/DBP |
| Cohort with cardiovascular disease ± risk factors | Variable | |

| | | |
|--|--|--|
| (Engvig, Fjell et al. 2012) | ↑mean cortisol | |
| (Manenschijn, Schaap et al. 2013) | ↓mean cortisol | |
| (Reynolds, Walker et al. 2010) | | |
| 17 controls and 19 in-patients with major depression(Pitts, Samuelson et al. 1995) | Blunted ACTH response to CRH Increased cortisol response to CRH | Hyperactivity seen in patients with major depression |
| Major depression | ↑mean cortisol | |
| (Gragnoli 2014) | ↑evening cortisol ↑inappropriately ACTH and CRH | |
| Lupus erythematosis(Weiner and Allen 1991) | | Hyporeactivity |
| Alzheimer's disease and mild cognitive impairment (MCI) | ↑basal cortisol throughout | |
| (Arsenault-Lapierre, Chertkow et al. 2010) | | |
| Poorer cognitive performance | ↓steep decline from early morning peak | |
| (Power, Li et al. 2008) | ↓CAR | |
| (Evans, Fredhoi et al. 2011) | | |
| Those with an an increased propensity for infections (Mason 1991) | ↑cortisol throughout | |
| Metastatic breast cancer (Abercrombie, Giese-Davis et al. 2004) | High peak cortisol, and then blunted decline | |

Conditions associated with ↑ evening cortisol
sleep disturbances; sleep
apnoea, chronic fatigue

→ metabolic
abnormalities (Spiegel,
Leprout et al. 1999)

Chronic fatigue (Demitrack, Low flat curves
Dale et al. 1991)

Post-traumatic stress Low flat curves
disorder (Yehuda, Giller et al.
1991)

Other factors affecting the pattern

As studies were reporting deviations of cortisol in both directions in the setting of chronic stress, a review by Miller, Chen and Zhou looked at the specific factors which may affect the deviation. (Miller, Chen et al. 2007) In particular they looked at

Time since onset of stress

The meta-analysis showed that there is a time dependant pattern, whereby as time passes, this activity diminishes, and cortisol secretion rebounds to below normal.

So they found, that the more months had elapsed, since first appearance of the stress, the lower a person's morning cortisol, daily volume, ACTH and post-dexamethasone cortisol. In our context, the duration of diabetes may therefore be important in predicting the pattern of cortisol release, as it could hypothesise that cumulative frequency of hypoglycaemia is greater in those with longer duration disease. If however, the stressor was still present (still experiencing hypoglycaemia), then daily cortisol output could be significantly higher.

However if the stressor was no longer present, morning and post-dexamethasone cortisol was significantly lower. However, the major limitation in these studies is that the majority are

looking at HPA through cross sectional studies. Although this gives us some indication of response of the HPA over time, it does not provide any information on the evolution of the HPA with time.

Nature of stress

This revealed that stress that threaten physical integrity produced a diurnal pattern that was high and flat. Morning cortisol was lower, but evening was much higher, leading to an increased daily output of cortisol. This can be justified, because in the presence of continued physical threats, the elevated HPA activity facilitates cognitive, metabolic, immunological and behavioural adaptations that maximise survival. However, a threat to the social self, was associated with significantly higher morning and evening cortisol levels, since people confronted with a social threat, need to acutely boost cortisol levels.

Controllability of stress

It is proposed that the controllability of a situation impacts on the stress that situation may conjure. Uncontrollable stress has been shown to elicit a flat, high diurnal pattern of cortisol secretion, manifested by a lower morning output, and higher afternoon/evening secretion (elevation in total daily volume of cortisol). However, other studies, have shown, when people encounter chronic stress that is uncontrollable, HPA activity decreases, and then behaviours such as withdrawal and disengagement emerge. However, if the stress was deemed to be controllable, the meta-analysis showed increased morning levels of cortisol, potentially preparing them to engage in active coping behaviours. Hence the patient's perception of hypoglycaemia, and in particular whether they felt it could be avoidable or not, may have an impact on the degree of HPA dysfunction.

Emotions elicited by stress

If a situation was thought to elicit shame, it was associated with significantly higher afternoon/evening cortisol, whereas if it involved loss, this had a flatter diurnal profile. This profile had a lower morning cortisol and a higher afternoon/evening cortisol. The shameful situation may be associated with troubling social interactions for the rest of the day, however in the situation of loss, there may be a context of social isolation or withdrawal from other social activities.

Individual psychiatric sequale

Psychiatric sequale of chronic stress has been shown to be a determinant of HPA activity with individuals showing markedly higher cortisol after the dexamethasone suppression test.

Perceived distress

Subjective distress has been found to be related to HPA activity perturbations. If a person had a higher level of distress, they showed greater total daily output, though morning levels were found to be lower.

Early life experiences

This has been particularly highlighted in studies on rodents, whereby early life experiences have been shown to program HPA functions at the genomic level, such that it remains unaltered throughout adulthood. (Liu, Diorio et al. 1997). A person's early experiences of hypoglycaemia may therefore influence their coping strategies, and particularly have an impact on the degree of HPA dysfunction later on in life.

So in summary, although we know that the HPA axis is activated during periods of stress, the axis is modulated by other factors relating to the stressful stimuli. The factors relate to the

nature, controllability and timing of the assessment in relation to the stress. In addition, the immediate sequale of the stress such as the emotions it elicits and the perception of the stress also appear to impact on the HPA axis.

Regulation of the HPA axis

Glucocorticosteroids (GC) is the end product of the HPA axis and is the main steroid hormone produced by the adrenal glands in man. The HPA axis is regulated by the supra-chiasmatic nucleus (SCN) which contains the body's "master-clock". (Abe, Kroning et al. 1979). The central clock is synchronised to the environment through signals from the retina. There are also peripheral "clocks" which are molecular oscillators in most cells and the pace of these clocks are set by the SCN (Dunlap 1999). Cortisol acts as a messenger between the peripheral and central clocks. The circadian output of the adrenal glands is now well known to play a part in resynchronisation of the internal environment with the external environment.

In mammals, it has long been known that there are dominant clock elements in the central nervous system and that the sleep/wake cycle of about 24 hours could be maintained even without any environmental clue. This was first described by Pincus where a diurnal variation of urinary ketosteroids was discovered in 1943. (Pincus 1943) Andrews and Folk discovered that circadian rhythms persisted in the isolated endocrine glands. (Andrews and Folk 1964) Discovery that removal of various endocrine organs did not abolish the circadian rhythm shifted attention to the CNS. In 1972, retinal fibres were found to not only project to the lateral geniculate nucleus, but also to a small nucleus in the anterior hypothalamus; the suprachiasmatic nucleus (SCN) (Hendrickson, Wagoner et al. 1972), and later on that year, it was shown that selective destruction of the SCN resulted in complete disappearance of the

circadian rhythm in adrenal corticosterone content (Moore and Eichler 1972) resulting in the understanding of the pivotal role of the SCN.

The circadian rhythm is organized in a hierarchical manner called the CLOCK system, and is a highly ubiquitous, conserved molecular system that synchronises endocrine daily rhythms to solar time through retinal afferents. The SCN lies at the top of this hierarchy, and is composed of densely packed neurons that have self-sustaining rhythmic capacity. (Herzog 2007) It has also been found that most mammalian cells also have their own circadian clocks (peripheral clocks), with a molecular makeup similar to that in the SCN neurons. The SCN acts as a co-ordinating centre for all the other peripheral clocks and thereby harmonizes the circadian rhythm throughout the organism. The SCN maintains continuous communication with the peripheral clocks, through multiple neural and hormonal signals. (Levi and Schibler 2007) SCN appears to influence peripheral clocks through the splanchnic nerve innervation (Jasper and Engeland 1997) to the adrenal gland and GC may be a hormonal link between SCN and the peripheral clocks and a mediator of the synchronicity of the circadian system.

The SCN provides input to the PVN which also receives afferent information from both the limbic areas of the central nervous system which are involved in detection of cognitive and emotional stress, and also the PVN receives information about physical stresses such as inflammation and hypotension from the brainstem. CRH and AVP containing parvocellular neurons in the PVN project to the median eminence of the hypothalamus, and CRH and AVP are released into the hypothalamic-pituitary portal circulation. They stimulate the corticotrophs cells in the anterior pituitary, which then releases corticotrophin (ACTH) into the general circulation. ACTH is formed from the cleavage of pituitary pro-opiomelanocortin

(POMC) by pro-hormone convertases. ACTH binds to the melanocortin-2 receptors in fasciculate zone of the adrenal cortex. This binding stimulates adenylyl cyclase, which activates protein kinase A resulting in gene transcription involved in glucocorticoid (GC) production. A series of biochemical steps result in GC formation from cholesterol. These are catalysed by cytochrome P450 enzymes which involve terminal hydroxylation reactions, which lead eventually to production of cortisol (predominant steroid in humans) and corticosterone. ACTH also stimulates release of oxytocin, adrenaline and noradrenaline which predominantly intensify the effects of CRH(Kudielka, Bellingrath et al. 2006).

Upon activation of GC onto its ligand, it undergoes a conformational change leading to the active component dissociating and entering the nucleus binding to the glucocorticoid response elements on the DNA. The rapid cycling of the receptor on and off the chromatin is thought to determine the ultradian rhythm(Conway-Campbell, Pooley et al. 2012).

GC acts on target tissues by binding to cytosolic receptors (glucocorticoid and mineralocorticoid receptors). There are two types of glucocorticoid receptor, with the low affinity Glucocorticoid receptor (type II) being only occupied during periods of high glucocorticoid secretion (mediating response to stress) and the high affinity mineralocorticoid receptor (MR) remaining in a bound state even in basal conditions, suggesting it is more involved with tonic HPA activity (De Kloet, Vreugdenhil et al. 1998). Upon binding, a conformational change occurs and the receptor is translocated to the nucleus, and influence gene expression. There is a negative feedback mechanism, with GC binding to the glucocorticoid and mineralocorticoid receptors in the brain turning off the HPA, and restoring a steady state. (Stratakis and Chrousos 1995) Negative feedback also occurs to the pituitary gland, PVN and hippocampus to inhibit ACTH release. There is a forward/feedback relationship between the pituitary gland and the adrenal cortex(Walker, Spiga et al. 2012), and it has been shown that a constant CRH concentration generated in the hypothalamus is

sufficient to generate an ultradian rhythm of both ACTH and glucocorticosteroids. However it is disrupted with higher levels of CRH suggesting that dose of CRH is more important than pattern in maintaining this ultradian rhythm. (Walker, Spiga et al. 2012) Loss of this ultradian rhythm in GC replacement, in patients for example with Addison's have been suggested to contribute to increased mortality in this patient group. However an acute administration of GC, has been shown to induce phase synchronisation in a wide range of peripheral clocks both in vivo and in vitro. (Balsalobre, Brown et al. 2000)

However the adrenal gland also appears to have an intrinsic mechanism which contributes to the diurnal rhythm of GC by controlling the daily variation in the adrenal sensitivity to ACTH. (Oster, Damerow et al. 2006). Specifically, steroidogenic acute regulatory protein (StAR) is thought to be the link between glucocorticoids synthesis from the adrenals and the CLOCK system (Son, Chung et al. 2011).

In summary, the HPA axis is regulated primarily by the SCN, which also receives input from retinal fibres. The SCN then synchronises peripheral clock mechanisms, with cortisol being the main messenger between the central and peripheral systems.

Dysregulation of the HPA axis

Acute prolonged periods of hypoglycaemia stimulates cortisol release from the adrenal glands, but the literature is sparse, with regard to impact of recurrent stimulation on various aspects of this axis. Stress is a culmination of dramatic stressful events but also the combination of the many events of daily life that lead to an increase in activity of physiological systems. This has been termed as wear and tear "allostatic load" (McEwen and Seeman 1999). It has been described as being reflective of the many life experiences, but also of genetic load, individual habits (diet and exercise) and developmental experiences,

which are important in the underlying physiological reactivity. Allostatic load occurs as a result of increasing external demands (see Figure 14) and excessive adaptive efforts, and this in turn can lead to dysregulation of the HPA axis, with impaired feedback and a disturbance in the diurnal patterns of cortisol release. This central dysregulation in turn has been shown to cause disturbances in other physiological systems such as the immune and cardiovascular systems, energy balance and fat deposition.

Dysregulation of the HPA axis has been hypothesized to be an early indicator of allostatic load (Abercrombie, Giese-Davis et al. 2004). Chronic stress has been shown in numerous studies to be associated with dysregulation of the HPA axis. Chronic dysregulation of the HPA axis is thought to be related with development of psychosomatic and psychiatric disorders.

We can glean some insight by looking at other systems of chronic stress models. For example, in a model of repeated stress involving repeated public speaking challenges, it was shown that there was lack of adaptation, resulting in prolonged exposure to the stress hormones (Kirschbaum, Prussner et al. 1995), with an inability to shut off the stress response even after the stress stimulus is terminated (Gerin and Pickering 1995). A further model suggests that chronic activation leads to exhaustion of the HPA (Seeman and Robbins 1994). The hippocampal region has been shown to be particularly vulnerable with many adrenal steroid receptors (McEwen and Magarinos 1997), wear and tear of this region, has been shown to be associated with dysregulation of the HPA axis and cognitive impairment (Sapolsky, Krey et al. 1986). The hippocampus is responsible for consolidating short term memory, however cortisol appears to suppress these mechanisms (Kirschbaum, Wolf et al. 1996). It is now becoming evident that chronic stress causes atrophy of pyramidal neurons in the CA3 region of the hippocampus (McEwen, Albeck et al. 1995). MRI studies have confirmed this hippocampal atrophy occurs in those with stress related disorders, such

as recurrent depressive illnesses and also in disorders with excessive cortisol, such as in Cushing's disease (McEwen and Magarinos 1997).

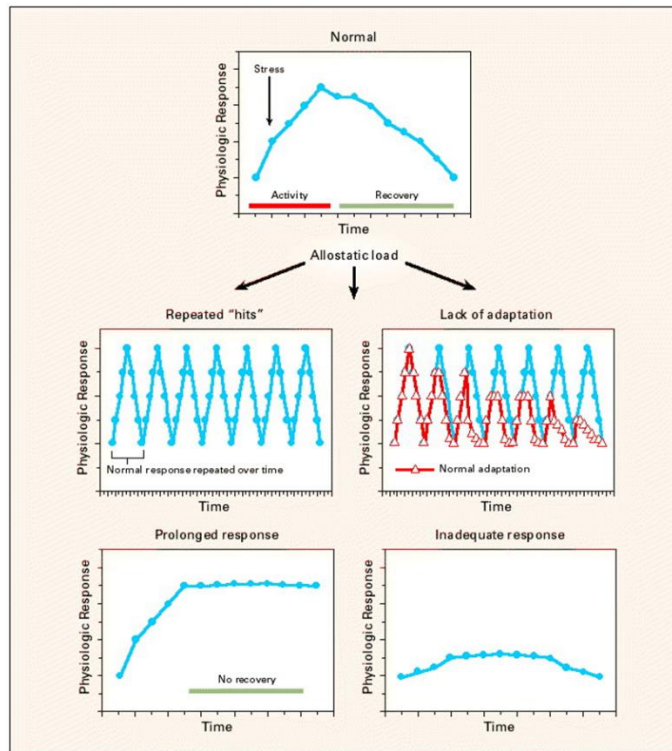
In contrast, short lived stress is usually accountable for reversible atrophy.

Apart from the system that controls the diurnal variation of GC, particular stressors are also able to activate the system. Here the HPA response often starts in the amygdala, which is an important part of the limbic system and is responsible for co-ordinating the negative emotional response to a stimuli which is deemed to be threatening. From here, the amygdala sends signals to the hypothalamus resulting in HPA activation. However, if there is continued stress and therefore high production of cortisol, GC has been shown to bind to the hippocampus and modulate brain function, thereby protecting the body from the effects of chronic hyper stimulation of the HPA axis (Graeff, Garcia-Leal et al. 2005).

Therefore, we know in summary that there is acute activation of the HPA system with stressful stimuli such as seen during hypoglycaemia. Chronic activation of stress systems or increasing allostatic load has in models of stress shown dysregulation of the HPA system. This dysregulation has presented in several ways in the literature as illustrated below (Figure 14). In severe cases of dysregulation, it has also led to hippocampal atrophy.

Figure 14; Allostatic load.

Top panel shows the normal allostatic response, with an initial stressor, sustained for an appropriate time, and then turned off. Remaining shows 4 abnormalities in this response; repeated hits from multiple stressor; lack of adaptation; prolonged response due to failure of negative feedback, and inadequate response with compensatory hyperactivity of other mediators.



Potential impact of dysregulation of the HPA axis on end-organs

There are now an increasing number of pathological conditions associated with a dysregulated secretion of glucocorticoids, and altered rhythmicity are frequently seen in many human diseases. (Chung, Son et al. 2011). It is still unknown whether the chronic dysregulation is a feature of the pathological process or is as a result of the process. We know that in the end, dysregulation leads to a disruption of carbohydrate and lipid metabolism, immune responses, cardiovascular activity particularly atherosclerosis of the carotid arteries (Dekker, Koper et al. 2008), mood and cognitive and brain functions. This is classically seen in Cushing's syndrome, with chronic exposure to glucocorticosteroids being

associated with obesity, insulin resistance, hypertension and hyperlipidaemia. Common psychiatric and/or somatic complex disorders, such as anxiety, depression, insomnia, chronic pain and fatigue syndromes (Pasquali, Cantobelli et al. 1993).

In specific, the uncoupling of the ACTH and cortisol which are necessary for the HPA axis regulation, has been shown to occur in many diseases states, and maybe as a result of autonomic or extrinsic causes. Disruption of the circadian rhythm such as chronic exposure to shift work, or sleep deprivation have shown to be linked to metabolic syndrome, characterised by impairment of carbohydrate and lipid metabolism, cardiovascular and haemostatic morbidity. (Garaulet and Madrid 2009)

Impact of glucose on the HPA axis

There are several similarities between the complications posed by diabetes and Cushing's syndrome, such as hypertension, immune suppression, myopathy and increased risk of depression, and this has questioned many investigators to suggest that diabetes could cause an abnormality in the HPA axis (Andrews, Herlihy et al. 2002). Cross sectional studies in patients with T1D have shown an increased levels of GC as well as increased urinary free cortisol levels (Roy, Roy et al. 1998). Possible explanations for this have included the effect of hyperglycaemia-induced stress and may be related to activation of the polyol pathway, which presents an alternative route for glucose metabolism, in the context of the adrenals producing high levels of aldose reductase (responsible for catabolism of GC in adrenal glands) (Matsuura, Deyashiki et al. 1996). Animal models of T1D have also shown an impairment in the GC negative feedback sensitivity, with higher levels of AVP, increased POMC expression and therefore increased levels of ACTH, with a high expression of MCR2

(ACTH receptors) in the adrenal glands, however a down regulation occurs of GR and MR in pituitary gland leading to the problems with negative feedback.

Insulin deficiency has also been shown to cause a hyper activation of the HPA system, however this deficiency through its actions on other hormones has also shown to be contributory. Namely both increases in glucagon and decreases in leptin has been shown to lead to the hyperactivity of the HPA axis (Chan, Chan et al. 2001). Glucagon has a central effect on the hypothalamus (Honda, Kamisoyama et al. 2012) stimulating eventual cortisol release, whereas leptin acts at all levels of the axis, inhibiting GC production (Roubos, Dahmen et al. 2012).

This hyper activation of the HPA axis, and therefore increased levels of glucocorticoids can pose difficulties in control of several diabetes related complications.

Impaired wound healing is a problem for many with diabetes. Hypercortisolaemia can impair collagen synthesis with reduced proliferation of fibroblasts. GC can also increase ROS production, and inhibit angiogenesis by reducing VEGF expression (Bitar 1998).

Although hyperglycaemia causes impairment of the ability of the body to fight against infection, the high circulating GC could also contribute to the increased infection rates with abnormalities in both neutrophil properties as well as defects in antibody responses (Dixon, Abrahamowicz et al. 2012). Hyperactivity of the HPA axis also leads to a hypertensive state, through activation of MR, with renal sodium retention, volume expansion and increase in BP and through up regulation of angiotensin II type 1 receptors on smooth muscle cells. GC also has an interaction directly with cardiac and vascular walls, which can facilitate plaque development (Baid and Nieman 2004). Finally HPA hyperactivity has been shown to be related to impaired hippocampus-dependent memory, synaptic plasticity and neurogenesis (Stranahan, Arumugam et al. 2008), and this hyperactivity (through stimulation

of catecholamines) may also explain the high incidence of depression in patients with diabetes(Muller, Zimmermann et al. 2003).

HPA hyperactivity seen in diabetes may also be compounded by acute hypoglycaemia (AH) which also activates various pathways involved in the regulation of the neuroendocrine stress response. There is currently very little in the literature on the effects of recurrent hypoglycaemia on the HPA in humans. Hypoglycaemia can be considered as a stressor, as it certainly activates the HPA.

Therefore this thesis seeks to explore the effect of recurrent hypoglycaemia/GV on the HPA axis with exploration of whether hypoglycaemia worsens the dysregulation of the HPA axis already assumed in patients with T1D. There is currently no clinical studies looking at this effect in a cohort with T1D.

CHAPTER 5

STUDY 2; Does adjunct DPP-4inhibitors in patients with T1D improve glucose variability, hypoglycaemic responses and glycaemic control?

Background

The loss of insulin secretion in patients with T1D leads to severe abnormalities in glucose metabolism that present as hyperglycaemia. Chronic hyperglycaemia leads to the development of micro- and macrovascular complications. Despite the variety of insulin replacement strategies and devices, there are numerous barriers to intensive insulin therapy in diabetes which include current limitations of insulin therapy (usually delivered subcutaneously by injection in a non-physiological manner), risks associated with hyperinsulinaemia, the risk of severe disabling hypoglycaemia, the need for daily and frequent capillary glucose monitoring and the psychosocial problems inherent in any chronic disease pathology, with most individuals with T1D still having suboptimal glucose control(Nathan, Cleary et al. 2005).

As a result of these limitations of insulin therapy, and since the rates of hypoglycaemia have remained unaltered over the last 20 years particularly in adults, there is currently a need to look at alternative adjunct therapies, to improve glycaemic control and reduce the burden of hypoglycaemia in patients with T1D. The last 90 years have focused on insulin as being the primary problem in T1D, however, it is now known that through multiple layers of evidence that there is a dysregulation of glucagon secretion. Of particular interest, is the therapeutic targeting of abnormal glucagon secretion in T1D? The pancreatic α -cell product glucagon acts primarily in the liver to stimulate glycogenolysis and gluconeogenesis and therefore to increase hepatic glucose production.

Abnormalities of the α cell

The importance of glucagon and its role in the catabolic manifestations of T1D, was largely overlooked for around 5 decades after the discovery of insulin. Suppression of glucagon in patients with T1D with agents such as leptin and somatostatin (Gerich, Lorenzi et al. 1975) was able to completely suppress the catabolic manifestations of total insulin deficiency.

Recent studies in animal models have also shown that T1D (induced chemically in transgenic mice) models which lack functional glucagon receptors do not develop diabetes, confirming the importance of glucagon in the development of hyperglycaemia (Lee, Wang et al. 2011).

It has also been shown in patients with T1D that there is a paradoxical increase in basal and post-prandial glucagon (Porksen, Nielsen et al. 2007) levels and this contributes to increased hepatic glucose output and glucose variability through a direct action of glucagon on the liver (Edgerton and Cherrington 2011). A recent study (Kramer, Borgono et al. 2014) showed that there was a significant paradoxical increment in glucagon ($p < 0.001$) following an oral glucose tolerance test regardless of prevailing glucose (either euglycaemia and hyperglycaemia). There was no difference ($p = 0.1$) in the rise of the glucagon in either of these conditions (when the ratio of change of glucagon was compared with change of glucose between the two states) in patients with longstanding T1D. This suggests that the normalisation of glucose (euglycaemic study) with exogenous insulin as in this study, did not correct this aberrant glucagon response, which therefore suggests limitations in the current intensive insulin therapy regimes. Notably elevations in glucagon levels are seen as early as the first year following diagnosis and appear to be correlated with blood glucose, with a 20% increase in glucagon release seen with every 10mmol/L increase in blood glucose, however it has been shown that this had little impact on overall HbA1c (Porksen, Nielsen et al. 2007).

On the contrary, patients with T1D have an inability to release glucagon in response to hypoglycaemia (Gerich, Langlois et al. 1973).

Therefore an agent which may be able to suppress the hyperglucagonaemia both in the fasting and the post-prandial phase, but not affect the counter-regulatory responses during hypoglycaemia, may have a potential impact on reducing the significant GV seen in this context in patients with T1D, and subsequently reduce the significant burden of hypoglycaemia (which is closely linked to GV).

DPP-4 inhibitors (DPP4-i); their potential utility in hypoglycaemia

In 1929, La Barre was the first to purify the glucose lowering component of gut extracts, and name it incretin (Zunz 1929). The incretins, both GLP-1 and GIP are secreted from the gut following intake of glucose or other nutrients. They act primarily through G protein coupled receptors, the GIPR and GLP-1R. The binding activates cyclic adenosine monophosphate, resulting in insulin secretion from the pancreas and also cause glucagon suppression from the cells. However both these incretins are rapidly deactivated by dipeptidyl peptidase-4 (DPP-4). We will therefore utilise the DPP-4 i (primarily for its glucagon suppressive action), which are currently used in the treatment of T2D, to determine whether it could be a suitable adjunct agent in patients with T1D.

Dipeptidyl-peptidase 4 inhibitors (DPP-4 i) are a class of orally active compounds designed to inhibit DPP4 enzyme activity, resulting in increased circulating levels of the incretins glucagon-like peptide 1 (GLP-1) and gastrointestinal peptide (GIP) (Thornberry and Gallwitz 2009). GLP-1 is an incretin that is secreted by ileal L cells in response to the presence of nutrients (carbohydrate, protein and lipid) in the lumen of the small intestine. Once in the circulation, GLP-1 has a half-life of less than 2 minutes, due to rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP4). GLP-1 acts in a glucose-dependent way to augment

insulin secretion while at the same time suppressing glucagon secretion, and additionally GLP-1 delays gastric emptying and increases satiety. There is evidence that GLP-1 infusion reduces fasting glucose concentrations (Creutzfeldt, Kleine et al. 1996) and post-prandial glucose levels (Dupre, Behme et al. 2004) in association with a reduction in glucagon in type 1 diabetes, although this is not evident in all studies (Raman, Mason et al. 2010). In a more recent study, Kielgast and colleagues (Kielgast, Asmar et al. 2010) demonstrated that infusion with GLP-1 in nine c-peptide negative type 1 diabetic patients clamped at 20 mmol/l significantly decreased the total area under the curve of glucagon by nearly 40%, and also suppressed arginine induced glucagon release.

GLP-1 receptor signalling pathway also enhances β -cell proliferation and regeneration and diminishes apoptosis, resulting in a greater β -cell mass (Mudaliar and Henry 2010). Based on these findings GLP-1 is a potential adjunct therapy in both c-peptide positive (β -cell preservation and proliferation) and c-peptide negative (α -cell suppression, reduced post-prandial glucose excursion, weight loss) type 1 diabetes (Thornberry and Gallwitz 2009) (Ellis, Moser et al. 2011, Farngren, Persson et al. 2012, Garg, Moser et al. 2013). Also reassuringly, during hypoglycaemia DPP-4 i does not further suppress glucagon secretion (Farngren, Persson et al. 2012) and in individuals with T2D might even improve glucagon counter regulation (Ahren, Schweizer et al. 2011).

These findings suggest that DPP-4 i adjunct therapy in T1D, could improve glucose variability by reducing both exposure to and duration of post-prandial hyperglycemia and hypoglycaemia. In T1D, increased GV is predictive of severe hypoglycaemia risk (Cox, Kovatchev et al. 1994), while recurrent hypoglycaemia leads to suppression of symptom and counterregulatory responses to subsequent hypoglycaemia; a clinical condition referred to as impaired awareness of hypoglycaemia (IAH) (McCrimmon and Sherwin 2010). This raises the intriguing possibility that DPP-4 i therapy in T1D may through reducing GV and

exposure to hypoglycaemia improve hypoglycaemia awareness and therefore prove a novel useful adjunct therapy in T1D irrespective of its ability to improve glycaemic control.

The available DPP-4 i can be divided into 2 main groups; those that are similar to the dipeptide structure of DPP4 substrates (sitagliptin, vildagliptin and saxagliptin) and those that are not (alogliptin and linagliptin). Nevertheless they are all competitive reversible inhibitors of DPP4. Differences exist in the binding processes; with sitagliptin, alogliptin and linagliptin forming non-covalent interactions with residues in the catalytic site, but vildagliptin and saxagliptin forming a reversible covalent enzyme-inhibitor complex with a slow rate of binding and dissociation, resulting in equilibrium between active and inactive forms. This slow equilibrium explains the longer duration of DPP4 inhibition activity in the latter group despite a shorter half-life. Vildagliptin and saxagliptin are cleared much quicker than sitagliptin resulting in shorter half-lives compared to alogliptin and linagliptin. A comparative study (sit, vild, sax, alo and lina) showed similar effect of DPP4-inhibition in vitro (95-97% for vilda and sit). Most of the DPP4 inhibition (90%) is attained within 15 minutes of inhibitor administration with a lesser amount (70-90%) sustained for 24 hours after administration. However there were differences in potency (IC_{50} 1nm for linagliptin vs 19nm (sita) 62nm (vilda) 50 nm (saxa). (IC_{50} ; conc of a drug required for 50% inhibition of enzyme). However used at the current therapeutic doses, the potencies are broadly similar.

Oral bioavailability as a result of negligible protein binding for the DPP-4 i is generally high (87% for sita, 85% for vilda and 67% for saxa), with DPP-4 inhibition seen within 5 minutes. Saxagliptin is metabolised by the liver through the P_{450} CYP3A4/5 system to produce 5 hydroxyl saxagliptin; BMS-510849 (which is also a reversible inhibitor of DPP4) and is renally eliminated as both a metabolite (21-52%) and as parent drug (11-29%). However 5 hydroxy saxagliptin is twofold less potent than saxagliptin but has greater selectivity than saxagliptin for DPP4 over DPP8 (948 fold compared to 391 fold) and DPP9 (163 fold vs 75

fold). Data suggest that the volume of distribution for DPP-4 i inhibitors is high, suggesting that they are widely distributed within tissues, however the highest concentration is found in tissues with the highest DPP-4. (E.g. intestines, kidney and liver). Most (75%) DPP-4 i are excreted primarily via the kidneys through a combination of glomerular filtration and active transport (Deacon 2011). DPP-4 inhibitors have a good safety profile. The terminal elimination half life ($t_{1/2}$) for both saxagliptin and its metabolite are 2.5 and 3.1 hours, respectively.

A recent meta-analysis and systematic review of the gliptin RCTs (Esposito, Chiodini et al. 2015) revealed that all available DPP-4 i shown a clinically meaningful reduction in blood glucose both in the fasting and post-prandial states with an average HbA1c reduction from baseline of -0.77% (95 CI -0.82 to -0.72), however there was high heterogeneity between trials. ($I^2=96\%$) with most of the total variance (58%) due to baseline HbA1c (34%) and fasting glucose (24%). Head to head trials of which there have only been 2 RCT have shown similar results between saxagliptin vs sitagliptin. Since their action is glucose-dependant, they have also been found to have a low risk of hypoglycaemia and weight gain (Ahren 2009).

Trials using DPP4-I in Type 1 Diabetes

A recent double blinded cross-over trial published showed in a group of 20 patients with T1D, sitagliptin (DPP-4i) given for 4 weeks dropped their mean glucose by 0.67mmol/L ($p=0.012$) and showed a reduction in the daily insulin dose (least-square means \pm sd = -0.051 ± 0.018 , $P = 0.012$), however there was no significant change in measures of GV (LBGI, MAGE, CV, SD) and in fact, there was a trend towards greater time spent in the hypoglycaemic ranges (Ellis, Moser et al. 2011). Although this trial was a cross-

over trial, there with no washout period incorporated in the design and there was a significant Hawthorne effect.

Furthermore a recent double-blind, randomized-parallel 20-week study exploring the mechanisms of DPP-4 i in T1D, revealed that sitagliptin given over a 20 week period, showed no significant reduction in 4hour postprandial glucagon reduction with consequently no change in HbA1c, insulin doses or weight, despite significantly elevated GLP-1 levels at 30minutes(Garg, Moser et al. 2013). The failure of DPP-4 i to demonstrate an effect in T1D, despite an increase in GLP-1, suggest that these agents may not provide sufficient glucagon suppression, to impact on both post-prandial peaks as well as more chronic glycaemic factors such as insulin doses and HbA1c.

A subsequent study with 141 patients with T1D given sitagliptin over 16 weeks(Garg, Moser et al. 2013), also showed that despite showing a post meal GLP-1 level which was much higher ($p<0.001$) and GIP levels which were lower ($p=0.03$), sitagliptin failed to find a lowering effect in glucagon AUC, A1c, insulin dose or weight despite in these patients. A non-significant trend in reducing hyperglycaemia was found only in those who were C-peptide +ve, the mechanism of which is unknown.

A recent open label study in 20 T1D patients, looked at the effect of combination therapy with sitagliptin/metformin (50/1000mg). Once again, although there was a reduction in HbA1c after a mean of 21 weeks, there was a return to baseline HbA1c after a mean of 49 weeks. However there was a persistent significant reduction in weight and insulin doses even at a mean of 49 weeks. It is possible that there was greater self-monitoring and self-adaptation during this study period or it may be purely the effect of the metformin as an insulin sensitizer rather than the sitagliptin(Vella, Buetow et al. 2010), which could also have explained these results.

Table 6 Summary of trial data of DPP-4 i use in T1D cohorts

| Trials | Agents used | Glycaemic effects | Non glycaemic effects |
|--|--|--|---|
| Open label ; CP -ve BH 8.7±1.3. (Giampietro, Giampietro et al. 2013) | Sitagliptin and metformin (46±19wks) | After 21wks; HbA1c 8.7±1.4 to 8.0 ±0.9% p<0.001 After 49 wks; HbA1c 8.7±1.3 p=ns | Sig dec in weight (mean weight loss 1.9±2.6kg p<0.001), fructosamine, LDL chol, insulin dose (0.73 to 0.60u/kg/d p<0.001) |
| Double blind, RCT, PC, CO CP -ve BH 7.5±0.55(Farngren, Persson et al. 2012) | Vildagliptin (4 weeks) | 120 min AUC of meal stimulated glucagon was sig lower (2.4 vs 2.6 p=0.022) No change in glucagon, adrenaline and cortisol during HHC. Sig drop in HbA1c from 58.1 to 54.7mmol/mol (P<0.001) No change in insulin doses | |
| Double blind, RCT, CO 20 subjects | Sitagliptin (4 weeks no washout period) | Sig drop in 2 hr PPG assessed on CGM worn over whole trial period. Sig drop in 24hr AUC glucose. Sig drop in HbA1c over 4 weeks. (-0.27% estimated diff p=0.03) Measures of overall GV (mean glucose, M100, J-index) significant drop Measures of within day and between day GV (SD, CV and MAGE) showed no sig change | No difference in weight. |

| | | |
|-------------------------------------|-------------------------|--|
| | | Trend for spending more time hypo (<3.1) p=0.08 |
| | | Sig drop in insulin doses LSM (- 0.051 p=0.012) |
| Open label, randomised study | Sitagliptin (1 year) | Sig drop in HbA1c 9.6 to 7.5% (p=0.0017). |
| C-peptide +ve | | Sig drop in insulin doses 0.84 to 0.4 (p=0.012) |
| N=6 | | No change in C peptide |
| (Hari Kumar, Shaikh et al. 2013) | | |

BH; Baseline HbA1c RCT: Randomized controlled trial. PC; Placebo controlled. CO. Cross over. CP; C peptide HHC; Hyperinsulinemic hypoglycaemic clamp. LSM; Least square means Mean±SD

A more potent drug; GLP-1R agonists

GLP-1R agonists however are a group of drugs, which are resistant to DPP-4 inhibition, and have a more direct (cf DPP-4 inhibitors) effect on both the beta and alpha cells to both increase insulin secretion and suppress glucagon secretion respectively. The results of GLP-1 agonists in T1D have been more positive, possibly as a result of a more powerful effect on the pancreas, but it has also been shown to have several extra-pancreatic effects (delay in gastric emptying, increase in brain satiety)

A study looking at 10 type 1 diabetic patients with residual β -cell function (C-peptide positive- all given liraglutide and insulin) and 19 without (C-peptide negative-randomly assigned to liraglutide plus insulin or insulin monotherapy) over a 4-week intervention period showed that total insulin dose decreased from 0.50+/-0.06 to 0.31+/-0.08 units/kg per day ($P<0.001$) in C-peptide-positive patients and from 0.72+/-0.08 to 0.59+/-0.06 units/kg per day ($P<0.01$) in C-peptide-negative patients treated with liraglutide but did not change with insulin monotherapy. HbA1c also decreased in both liraglutide-treated groups, but this did

not reach significance. At the same time, weight loss with liraglutide was common during treatment (mean [range] -2.3+/-0.3 kg [-0.5 to -5.1]; $P<0.001$).

In a longer-duration open label, proof of concept, study conducted by Varanasi et al (Varanasi, Bellini et al. 2011), 24-weeks liraglutide and insulin therapy in type 1 diabetes in addition to beneficial effects on weight and mean HbA1c, resulted in significant reductions in mean fasting and mean weekly glucose concentrations, as well as reduction in glycaemic variability measured by coefficient of variation (cv). Conversely, blocking endogenous GLP-1 receptors has been shown to increase post-prandial glucagon levels (Kielgast, Holst et al. 2011)

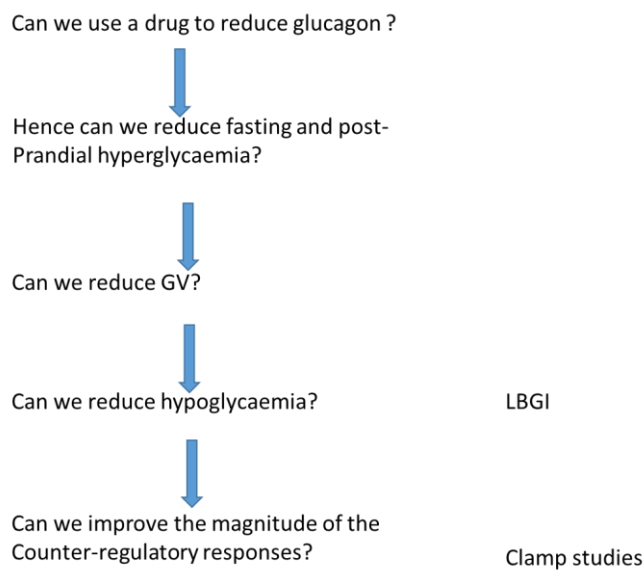
A recent oral presentation(Kuhadiya 2014) at the American Academy of Clinical Endocrinologists presented findings on the first RCT on the effects of adjunct liraglutide therapy in those with T1D. In 72 patients with T1D (mean HbA1c $7.57\pm0.09\%$) of at least 1 year, 54 patients received either 0.6mg, 1.2mg and 1.8mg of liraglutide daily for 12 weeks; 18 patients were randomized to placebo. The 0.6mg and placebo showed no benefit, apart from weight loss of 6lbs in the 0.6mg group. In the 1.2mg and 1.8mg, benefits were seen as compared to placebo, in mean change in average blood glucose (-10 ± 2 and -10.0 ± 1 mg/dL $p<0.0001$ vs placebo), in HbA1c respectively (-0.78% ($p<0.01$ vs placebo) and -0.42% ($p=0.39$ vs placebo). There was also a significant reduction in insulin doses (12.4 ± 3.9 units, 10.0 ± 2.3 units) and a significant improvement in diabetes specific quality of life in both the higher doses. In addition, those on the 1.8mg dose, showed a significant improvement in systolic blood pressure.

A further study(Sarkar, Alattar et al. 2014) showed that 24 weeks of exenatide also resulted in a significantly lower post-prandial glycaemia, despite a reduction in meal time insulin doses in patients with long duration T1D. This is particularly significant as post-prandial

glycaemia is thought to be a strong risk factor for cardiovascular disease(Ceriello 2005). Furthermore this study(Sarkar, Alattar et al. 2014) showed 85% of participants having a marked improvement in their insulin sensitivity, as assessed through a euglycaemic clamp. Although this has been shown in animal models, the mechanism in improvement of whole body insulin mediated glucose utilization has not been fully understood (Gedulin, Nikoulina et al. 2005).

Although the evidence points to a more powerful glucagon suppressive effect with GLP-1R agonists, STUDY 2 utilised the less potent DPP4-I because of the difficulties in producing a placebo-controlled product for the injectable former therapy and also the ease for the patient of an oral medication. DPP4-I were therefore used for its ability to reduce both fasting and post-prandial glycaemic load, and consequently GV. The association between GV and hypoglycaemia, meant that this therapy could in turn improve the CRR and therefore hypoglycaemia awareness. (See figure 15)

Figure 15 ; Proposed mechanism for STUDY 2.



Non-glucose effects of incretins ; effect on vasculature

The endothelium is an effective transducer of signals between the circulation and the vessel wall, and responds through production of a wide range of factors which regulate vessel tone, adhesiveness, growth and coagulation (Luscher 1990). A change in the function has been shown in several studies to predate the development and progression of atherosclerosis (Juonala, Viikari et al. 2004, Halcox, Donald et al. 2009). Hyperglycaemia has shown to reduce the migratory capacity of endothelial cells to areas of need in order to restore good endothelial integrity. A recent study also showed that endothelial progenitor cells (EPC) when cultured in high glucose had a mitochondrial impairment and autophagy.

The bone marrow is key in producing endothelial progenitor cells (EPCs) (Fadini, Agostini et al. 2007). Maintaining a good level and function of EPCs is crucial for good cardiovascular health. Levels are considered a good biomarker for coronary and peripheral artery disease, with a low EPC level predicting future cardiovascular outcomes (Fadini, Losordo et al. 2012). There is now a body of evidence that there is both an impairment of function and reduction of

EPCs in both T1D and T2D (Menegazzo, Albiero et al. 2012). This is in part due to diabetes affecting the structure and function of the bone marrow (Fadini, Boscaro et al. 2010).

The DPP4 enzyme cleaves a dipeptide from the N terminus of several proteins, and therefore not only is GLP-1 one of the main substrates for DPP4 inhibition, but it has come to light recently that SDF-1 α (CXCL12) is also another important substrate (Ou, O'Leary et al. 2013). SDF-1 α binding to its receptor (CXCR4) stimulates the bone marrow to produce endothelial progenitor cells (EPCs). The complex then undergoes homodimerization and then interacts with inhibitory G α i, which is then phosphorylated by JAK2/3 kinase and STAT factors. When DPP4 binds to SDF-1 α , it has been shown to impair its capability to trigger CXCR4, inhibiting its chemotactic function (Proost, Struyf et al. 1998). So theoretically if DPP4-inhibitors can block SDF1- α breakdown, then they could exert a positive effect on vascular progenitor cells. Indeed this was shown in a recent study where sitagliptin was given for 4 weeks, and showed a significant increase in SDF1- α and also in endothelial progenitor cells (EPC)(Fadini, Boscaro et al. 2010). There has been much debate as to how an EPC is defined. CD34+ is expressed on most EPC. However a recent study looking at the effect of sitagliptin on EPC in patients with T2D, showed a 2 fold increase in CD34+KDR+, but no change in CD34+. This was explained because SDF-1 α receptor CXCR4 was only expressed on 17% of CD34+ and on 63% of CD34+KDR+ cells.

Furthermore a recent study, showed that during both acute hyperglycaemia and acute hypoglycaemia, there is a significant increase in MMP-9 (possibly through generation of oxidative stress (also increase in 8-iso-PGF2 α), however simultaneous infusion of GLP-1 was able to counter-act this effect in both scenarios(Ceriello, La Sala et al. 2015). Therefore an agent such as DPP-4 i may have a further therapeutic benefit, through increasing endogenous GLP-1 on reduction of oxidative stress, and potentially on cardiovascular disease.

DPP-4inhibitors (DPP-4 i) (des-fluor-sitagliptin, (Matsubara, Sugiyama et al. 2012) sitagliptin(Shah, Kampfrath et al. 2011) vildagliptin(Maeda, Matsui et al. 2012)) has also shown to inhibit inflammatory cytokines such as TNF- α , interleukin (IL-6), IL-1 β and MCP-1, and cause up regulation of endothelial NO production and also to suppress the NF- κ β in experimental animal models.

Therefore, focusing on therapies such as DPP-4 i to both improve function and number of EPCs, with its additional possible anti-inflammatory role, as well as increasing GLP-1 may potentially offer additional benefits and aid in reducing macrovascular burden of T1D.

Methods

We designed an investigator-funded 12-week double-blind, randomized, cross-over study in individuals with established c-peptide negative T1D to determine whether DPP-4 inhibition, through primarily its glucagon suppressive effect was able to reduce insulin requirements and improve overall glucose variability, and would subsequently reduce frequency and exposure to hypoglycaemia, therefore improving the magnitude of the counterregulatory symptom and hormone responses during subsequent insulin-induced hypoglycaemia.

This was a single center, randomized, double-blind, placebo-controlled randomized trial.

Ethical approval was obtained from an independent research ethics committee and the Medicines Healthcare Products Regulatory Agency (MHRA)). The study was carried out in accordance with the Declaration of Helsinki, and written informed consent obtained from all participants before inclusion in the study. All participants were identified using the Scottish Diabetes Research Network (SDRN), and the study took place at the Clinical Research Centre, Ninewells Hospital, Dundee. (Ethics REC No; 12/SS/0125 and Clinical Trials.gov;

NCT 01922817. Adult subjects (n=14) with well-controlled c-peptide negative T1D >5 years duration were recruited and underwent medical screening.

Criteria are detailed below

Table 7 Inclusion and exclusion criteria for the DPP-4 i study

Inclusion criteria

- Type 1 diabetes with greater than 5 years disease duration
- HbA1C<10%
- Age 18 and over
- Current use of intensive insulin therapy (injections or pump)
- BMI 19-35 kg/m²
- Ability to give written informed consent to participate in the study

Exclusion criteria

- Previous history of pancreatic disease/cancer
- Significant renal disease eGFR <50 ml/min
- Significant microvascular disease
- Personal/family history of Medullary thyroid cancer
- Personal/family history of MEN Type 2
- Moderate/Severe hepatic impairment (Child Pugh B,C)
- Pregnancy/Breast feeding
- History of epilepsy/hypoglycaemia induced seizure
- Those on any other hypoglycaemic drug apart from insulin for their diabetes.
- Currently on CYP3A4 inducers like carbamazepine, dexamethasone, phenobarbital, phenytoin and rifampicin
- Currently on CYP3A4 inhibitors like ketoconazole, diltiazem
- Less than 30 days since participation in another drug trial or longer depending on the drug half-life.

Baseline demographic and information on current diabetes management was collated.

Screening bloods (renal and liver enzymes) and thorough review of medical notes was conducted to ensure that the participant could be included. Consenting participants had an initial 3-4 week baseline period where they underwent two blinded continuous glucose monitoring (CGM) periods for at least 5 days (one at the start and one at the end).

The first blinded CGM (iPRO) was used for education purposes – following this each participant had their insulin, dietary and exercise regimes completely reviewed by myself for consistency and carbohydrate ratios reviewed by a single dietician. Treatment of hypoglycaemia was re-iterated with an emphasis on quick recognition and treatment of all hypoglycaemia episodes. These were all done in a one-to-one manner. The participant would then have an opportunity to optimize their diabetes management. A second blinded CGM was performed after a minimum of 3-4 weeks and the data from this was used as a baseline for calculation of glycaemic variability (GV) indices prior to entry into the drug treatment phase. During each CGM, participants were required to fill in the iPRO blood glucose-recording diary for calibration purposes during the 5-7 day monitoring period. This involved checking blood sugars at least 3 times a day prior to meals and an additional reading prior to bed. In addition, participants were also encouraged to check blood sugars during all symptomatic hypoglycaemia episodes, and to record all blood sugars below 3.5mmol/L (frequency of hypoglycaemia measures). The data sheet from the iPRO web based software was exported to EasyGV (Hill, Oliver et al. 2011), an excel-enabled workbook. This program uses macros to calculate 10 different measures of glycaemic variability from continuous glucose monitoring data using a simple interface. For the purposes of this study, we focused on Low Blood Glucose Index (LBGI) and Average Daily Risk Range (ADRR). The former was chosen as an index reflecting hypoglycemic stress during the monitoring period (Kovatchev, Cox et al. 1998) and the latter, because it is equally sensitive to both hypoglycaemia as well as hyperglycaemia (due to log transformation of original values) and is a good marker of both extremes of GV (Kovatchev, Otto et al. 2006). HbA1c, insulin doses and weight were also recorded prior to the first treatment phase.

Subsequently, subjects were enrolled into Group 1 (Gp 1) or Group 2 (Gp2) using a randomized block design. Subjects were randomized in blocks of 4 using a computer generated randomization sequence generator. Seven subjects were in each treatment sequence. Sequence A received placebo for the first 12 weeks, before receiving the DPP-4 i for the second arm. Sequence B was in reverse order to Sequence A. All subjects were advised to continue their usual diabetes, dietary and exercise regime during the entire trial. Subjects were contacted on a weekly basis for the first month, and then monthly thereafter. During each contact, adverse events were recorded and advice provided as required on insulin dose adjustment.

Subjects were provided with a single daily oral 5mg dose of the DPP-4 inhibitor saxagliptin (Onglyza ®, Bristol Myers Squibb) or placebo for 12 weeks. Both placebo and Saxagliptin were encapsulated to ensure they were identical in appearance. At the end of each 12-week period the subjects underwent a further period of blinded CGM (at least 5 days), blood samples were taken and each subject underwent a hyperinsulinemic hypoglycemic clamp study to assess the magnitude of their counter-regulatory responses. Participants had at least a 2-week washout period before entering the second arm of the trial.

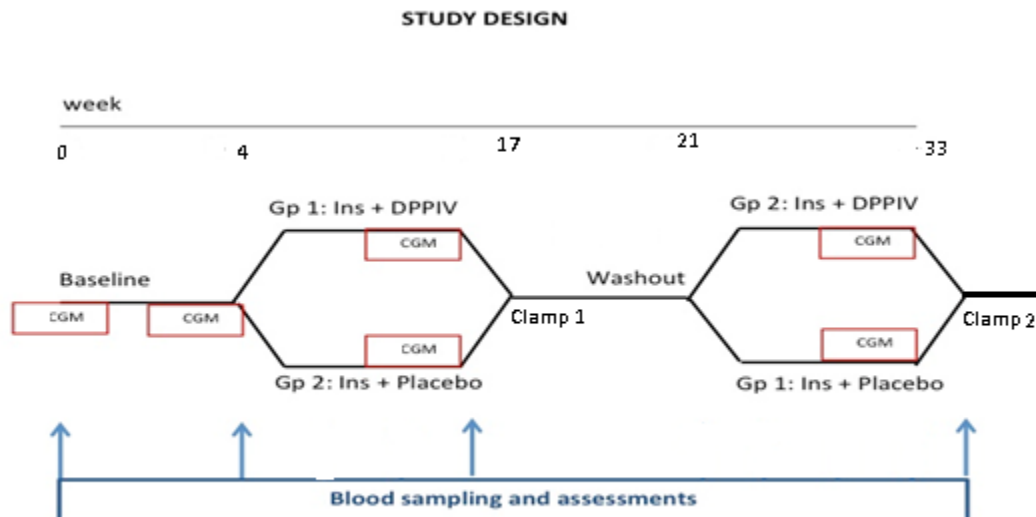


Figure 16; Diagrammatic representation of DPP-4 i study design

Single step hyperinsulinemic hypoglycaemic clamp

Overnight-fasted subjects reported to the Clinical Research Centre, Dundee at 8.00am. All subjects were asked to avoid hypoglycaemia (<3.5mmol/L) in the last 24 hours prior to the clamp study and to reduce their long acting insulin by 10-20% and this was subsequently confirmed by CGM (this period was excluded from final analysis of GV). A cannula was inserted into the non-dominant hand, and placed in a heated box (50-55°C) to obtain arterialized venous blood. A further cannula was inserted into the dominant antecubital vein of the contralateral arm. Insulin was started at a priming dose of 15u/hr until a blood glucose of 7.0 mmol/L was reached, and then insulin was maintained at a dose of 1.5mu/kg/min. Glycaemic plateaus were achieved through bedside measurement of blood glucose (Analox GM9D, Analox instruments, London, UK) every 5-10 minutes, and using a variable 20% dextrose infusion. Subjects were initially maintained in the euglycaemic range (between 4-6mmol/L) for 40 minutes, prior to hypoglycaemia (2.5mmol/l) being induced and subsequently maintained for 85mins. Blood samples for determination of insulin, adrenaline,

noradrenaline, and glucagon were drawn in triplicate during the baseline period, and then every 20 minutes during the hypoglycemic phase. Blood pressure and pulse rate were measured every 10 minutes (Accutorr Plus Monitor, Datascope Corp., New Jersey, USA).

Blood sampling and analyses

Samples were centrifuged to separate the plasma within 2 hours, and then stored at -80° c prior to assay. Hormones (Insulin-RIA-Diasorin; CV inter -6.7%, intra -5.8%), (Glucagon-RIA-MilliporeUK; CV inter 4.9%, intra 8.8%), (Adrenaline-EIA-Alpco; CV inter 22%, intra 16%), (Noradrenaline-EIA-Alpco; CV inter 16%, intra 22%) were measured by ELISA, and samples were analyzed in duplicate according to the manufacturer's instructions.

Symptoms and cognitive function tests

Subjects rated hypoglycaemia symptoms three times over the 40 minute euglycaemic period and every 20mins during the hypoglycemic plateaus. Symptoms were scored on a validated questionnaire, the Edinburgh Hypoglycaemia Scale, scoring from 1 (not at all) to 7(very severe) on a visual analogue scale. Symptoms included autonomic (hunger, palpitations, sweating, shaking), neuroglycopenic (drowsy, confused, odd behavior, speech difficulty, inco-ordination) and non-specific symptoms (nausea, headache) (Deary, Hepburn et al. 1993).

Cognitive function was assessed along with symptoms using Trail Making B(TMB)(Kortte, Horner et al. 2002) and Digit symbol substitution test (DSS) tasks, which are known to be sensitive to hypoglycaemia (Wechsler 1981). To minimize learning effects, all subjects had practiced both tasks. (5-7 days prior to the clamp study and also twice at the start of the clamp study)

Statistical analysis

Data are reported as mean \pm SEM. The pre-specified primary outcome was the adrenaline counter-regulatory response during insulin-induced hypoglycaemia following 12 weeks of saxagliptin treatment vs. placebo. Prior power calculations indicated that 12 subjects were needed for a matched analysis, with 80% power to detect a difference in change of 450pmol/L (Amiel, Sherwin et al. 1988) with SD of 500 and an alpha of 0.05, two sided. Additional subjects were recruited to account for a potential 25% dropout rate. Secondary outcomes included insulin requirements, HbA1c, glucose variability indices, frequency of hypoglycaemia, hypoglycaemic awareness, and glucagon response during hypoglycaemia. Statistical analyses were conducted using Graphpad Prism 6 and $p < 0.05$ was considered statistically significant. Normally distributed data were compared using paired samples t tests, while non-normally distributed data were compared using the Wilcoxon signed rank test. Repeated measures ANOVA was used to determine differences in other parameters measured over time, with t -testing used to localize effects where indicated. No order effects were noted in any of the subsequent analyses.

Visits structure

V1; Informed consent was taken on the first visit (V1). Screening was performed. (See Inclusion and Exclusion Criteria in Table 3). History of frequency of hypoglycaemia alongside questionnaires to assess hypoglycaemia awareness (Gold) will be assessed. A comprehensive history of their diabetes will be taken including current insulin doses. I will also document their past medical history and general demographics (BMI, Sex, Ethnicity). Baseline bloods will be taken (FBC, UE, LFT, Amylase, Lipids, and HbA1c). A continuous glucose monitor (CGM) will be fitted and participant encouraged to fill simultaneously a food and insulin diary.

V2; 2-7 days after V1, the CGM will be reviewed. General dietary and insulin advice will be given. A dietician will also be present. Participant will be encouraged to make appropriate changes over the following 4 weeks. The 4-week baseline period is designed to familiarize the participants with CGM and to optimize diabetes management prior to entry into the trial. The first CGM will be predominantly used for educational purposes.

V3; 3-8weeks after V2, a 2nd CGM would be fitted.

V4; 2-7 days after V3, CGM will be reviewed. A review of hypoglycaemia frequency will be recorded. This CGM will be used for assessment of glucose variability prior to entry into the treatment phase. The investigational medicinal product (IMP) will be issued to the participant.

Follow up; I made telephone contact with the participant on a weekly basis for the first month, and then monthly contact after that. This will be used both as an opportunity to reduce insulin if the participants are having increasing hypoglycaemia episodes and also to document any adverse events.

V5; 12-14weeks after V4; Frequency of hypoglycaemia and insulin doses would be documented. Baseline bloods would be repeated. A repeat CGM would be performed. The participant will be familiarised with the cognitive tests to be performed during the hyperinsulinemic hypoglycaemic clamp study a few days later.

V6; 2-7 days after V5; CGM will be used to assess glucose variability post treatment 1.

Pt will undergo the single step hyperinsulinemic clamp study. At the end of the study, participants will then be given a meal, and asked to take their short acting insulin. After their meal, we will make sure their parameters such as BP, pulse, blood sugars are in an acceptable range, and that the participant feels back to normal before considering discharge.

There will then be at least a 2 week washout period.

V7; Subjects will be given the IMP (placebo/drug), and compliance will be reinforced.

Telephone contact with the participant will be made on a weekly basis for the first month, and then monthly contact after that. This will be used both as an opportunity to reduce insulin if the participants are having increasing hypoglycaemia episodes and also to document any adverse events.

V8 and V9; will be similar to V5 and V6.

Results

Eighteen subjects with T1D were screened, with fourteen (8 male, 6 female) Caucasian subjects completing the two arms of the trial. The consort diagram is shown in Figure 9.

Mean age of participants was 42.9 (3.3) years. Mean weight was 74.1(3) kg. All participants had C-peptide –ve (<0.10nmol/L) T1D, with a mean duration of disease of 20.5 (2.7) years with relatively well controlled disease (mean HbA1c 64 (2) mmol/mol. Mean insulin doses were long acting insulin 27 (4) and short acting insulin 28 (4) units. Median (IQR) baseline Gold score was 3.0 (2-4). (See table 8) Compliance with study drug was high in both arms of the trial (placebo and saxagliptin arms, 94.4 and 91.8% respectively).

Figure 17 Consort diagram for the DPP-4 i study

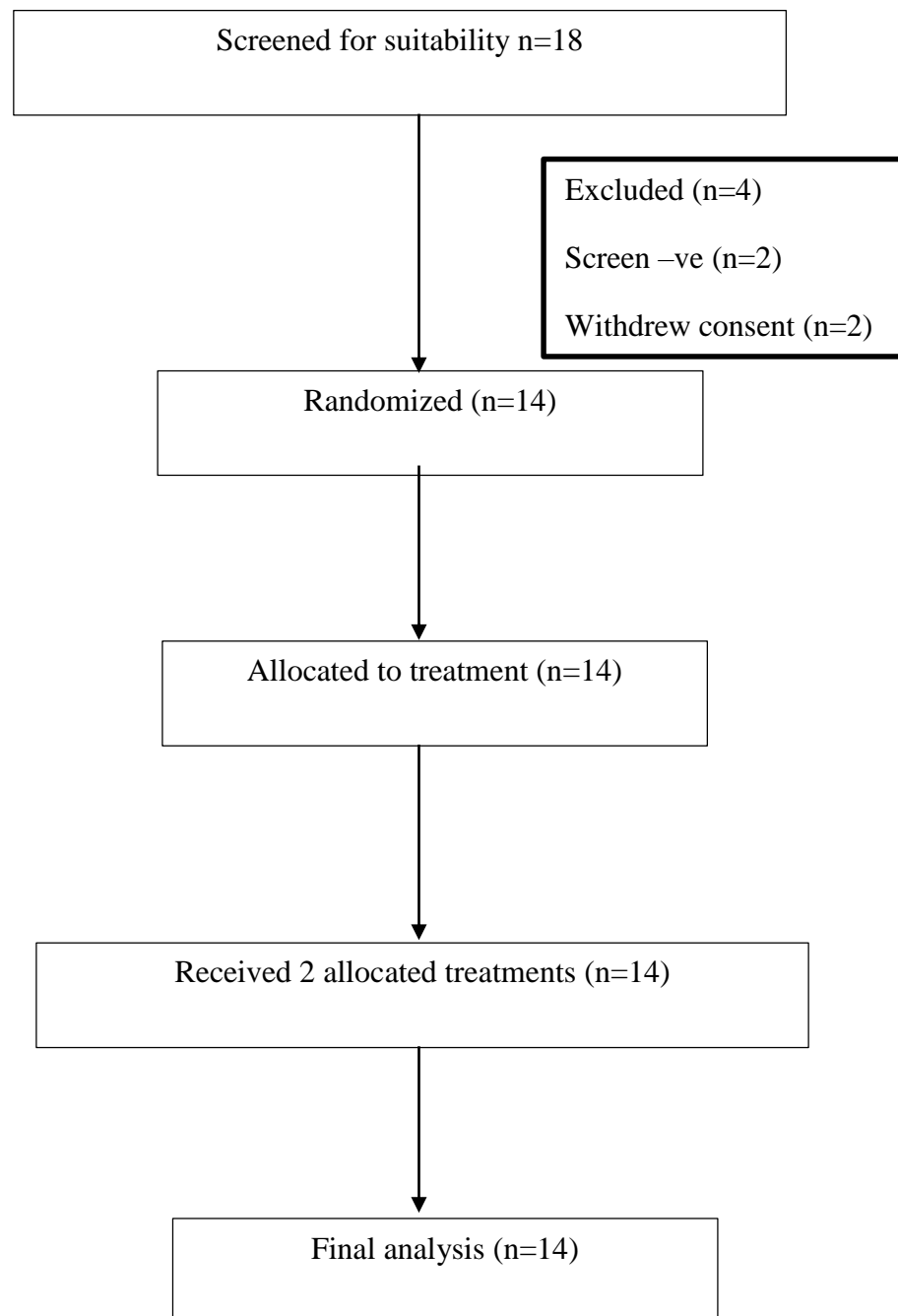


Table 8; Baseline clinical and biochemical characteristics for STUDY 2

| | |
|---|------------------------------|
| Median (IQR) age (yrs.) | 44.5 (14) |
| Median (IQR) weight (kg) | 72.3 (13.4) |
| Median (IQR) duration of diabetes (yrs.) | 17.5 (15.5) |
| Median (IQR) Gold Score | 3 (1.8) |
| Median (IQR) HbA1c (mmol/mol/%) | 63mmol/mol/7.9% (7.5) |
| Median (IQR) insulin doses | |
| -long acting (u) | 28 (18) |
| -short acting (u) | 25 (18) |

Glycaemic control, hypoglycaemia frequency, hypoglycaemia awareness and body weight

There was no overall effect of saxagliptin on glycaemic control [HbA1c F (1, 11) =2.49 p=0.14],

Or daily insulin dose [F (1, 11) =0.069 p=0.80].

Table 9 Measures of glycaemic control and glucose variability following 12-weeks adjunct therapy with DPP-4 i (Saxagliptin) or placebo in subjects with type 1 diabetes. Glucose variability measures recorded using continuous glucose monitoring assessments in the final week of the trial. Values shown as mean (SEM).

| | Variable | Placebo | Saxagliptin |
|-----------------------|-------------------------|------------|-------------|
| Glycaemic control | HbA1c (mmol/mol) | 66 (2) | 65 (2) |
| | Total insulin dose (iu) | 60 (8) | 56 (7) |
| Glycaemic variability | LBGI | 6.1 (1.6) | 6.1 (1.8) |
| | HBGI | 12.8 (1.6) | 13.5 (1.9) |
| | ADRR | 12.3 (1.9) | 12.3 (1.7) |
| | Mean glucose | 9.7 (0.6) | 10.2 (0.6) |
| | SD | 3.6 (0.2) | 3.7 (0.3) |

During each treatment phase the change in HbA1c was small (0.3mmol/L with saxagliptin and 1.6mmol/L with placebo) and did not differ significantly between groups ($p=0.61$). In addition, no overall effect of saxagliptin on self-reported hypoglycaemia frequency [$F(1, 11) = 0.393$ $p=0.54$] or hypoglycaemia awareness [$F(1, 11) = 3.43$ $p=0.09$] was seen. There was no effect of saxagliptin on weight [mean increase of 0.24kg with saxagliptin and 0.07kg with placebo; $F(1, 11) = 0.40$ $p=0.54$].

Glucose variability

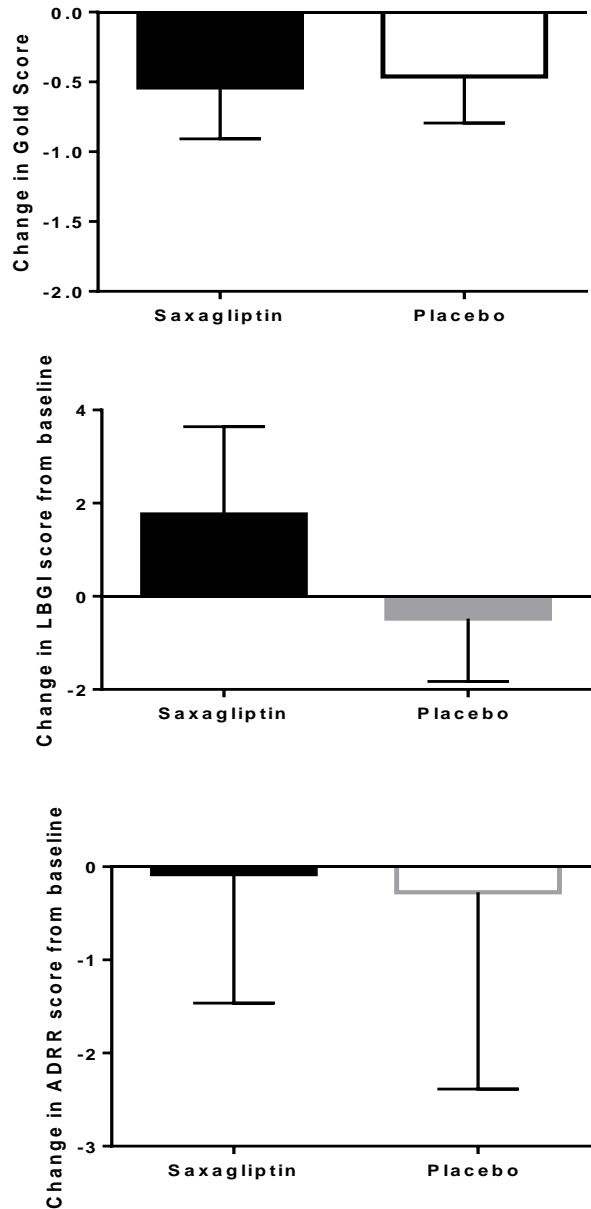
No significant effect of saxagliptin adjunct therapy was seen on CGM measures of mean or

Standard deviation of glucose or on the principal measures of LBGI [$F(1, 9) = 0.418$

$p = 0.534$] or ADRR [$F(1, 9) = 0.365$ $p = 0.365$] (see Table 4) (see Figure 9)

Figure 18. Effect of saxagliptin on Hypoglycaemia Awareness, Low Blood Glucose Index and Average Daily Risk Range.

Non-insulin adjunct therapy with Saxagliptin in c-peptide negative type 1 diabetes had no effect on (A) Hypoglycaemia Awareness, (B) Low Blood Glucose Index (LBGI), (C) Average Daily Risk Range (ADRR). Saxagliptin shown by black bars, Placebo by white bars. Values shown as Mean \pm SEM.



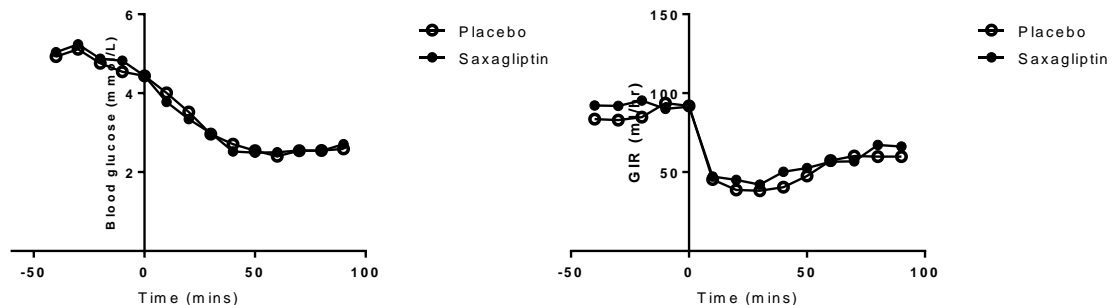
Hyperinsulinemic hypoglycaemic studies

Glucose profiles during the hyperinsulinemic clamps studies were also well matched with no

Effect of treatment [$F(1, 26) = 0.00$ $p = 0.96$]. (Fig 11a/b)

Figure 19; Blood glucose and Glucose Infusion Rate during STUDY 2 clamp studies

Blood glucose (a) and GIR (b) was comparable in both hyperinsulinemic hypoglycaemic clamp with placebo and with saxagliptin



Glucose infusion rates (GIR) required to maintain the hypoglycaemia plateau were also comparable in the two treatment groups [$F(1, 26) = 0.23$ $p = 0.64$] (Figure 10b).

Plasma adrenaline increased with time over the clamp period [main effect of time, $F(6, 156) = 40.36$ $p < 0.0001$]. However there was no effect of treatment [$F(1, 26) = 0.02$ $p = 0.89$] and

There was no time X treatment interaction [$F(6, 156) = 0.17$ $p = 0.98$]. The AUC of the Adrenaline responses were also similar between groups [25,775 vs. 24,454, Placebo vs. Saxagliptin, respectively, $p = 0.76$] (Figure 11a). No significant effect of either hypoglycaemia or treatment was seen on the glucagon response to hypoglycaemia ($p = \text{ns}$; Figure 11b)

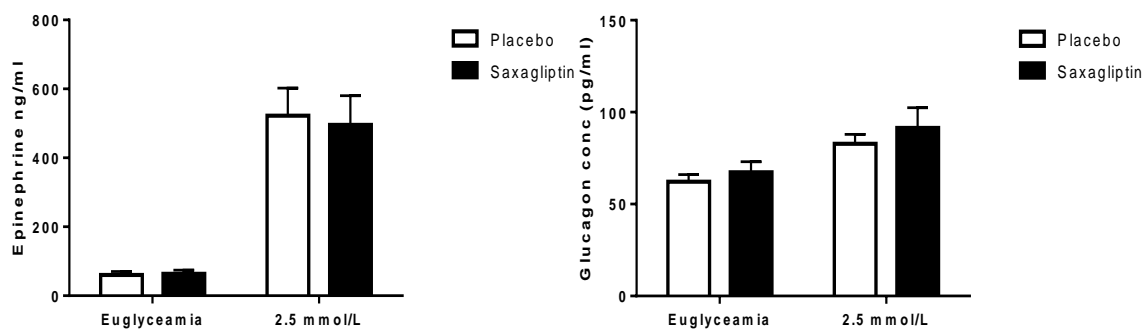


Figure 20; Comparison of Epinephrine and Glucagon responses in placebo/saxagliptin clamp studies.

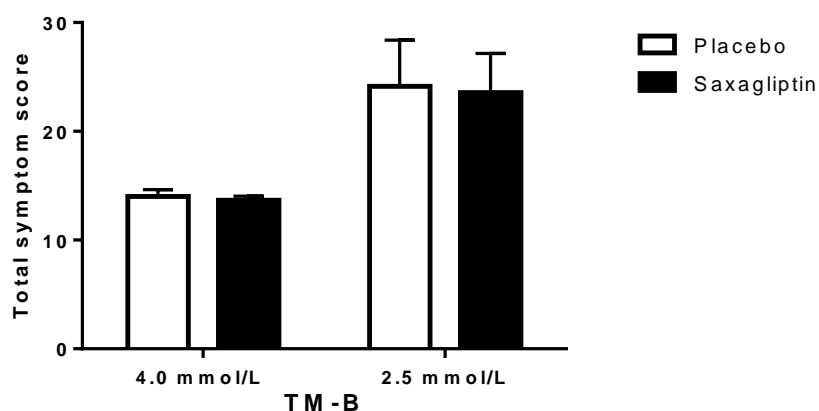
During the hyperinsulinemic hypoglycaemic clamp studies, there was no significant differences in adrenaline and glucagon secretion with saxagliptin

Consistent with the hormonal responses, subjects did not report any differences in their total symptom scores during hypoglycaemia between the two treatment arms [26 (4) vs. 28 (3), Placebo vs. Saxagliptin; $p=0.38$], or between autonomic symptoms [12 (1) vs. 13 (1), Placebo vs. Saxagliptin; $p=0.36$] (see Figure 12a). The two groups also performed similarly on cognitive tasks during hypoglycaemia: TMB [37 (6) vs. 37 (8) s; $p=0.96$] or DSS [67 (4) vs. 62 (4); $p=0.16$] both Placebo vs. Saxagliptin, respectively (see Figure 13 b and c).

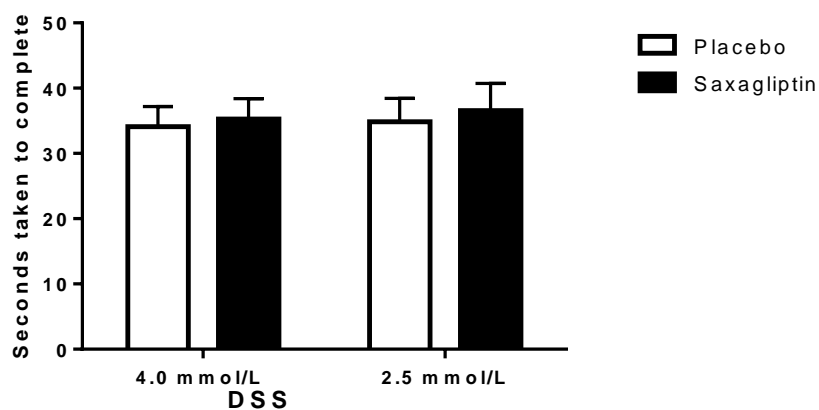
Adverse events

No serious adverse events were reported during the trial. Other adverse events reported were infrequent (<10%), mild and did not differ with placebo or Saxagliptin therapy.

a.



b.



c.

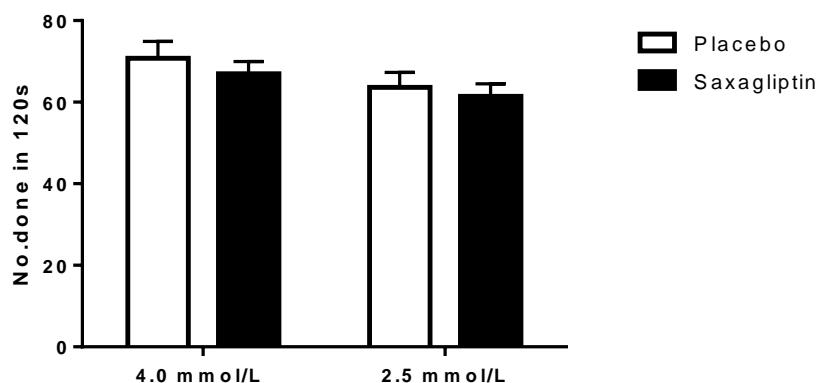


Figure 21; Effect of saxagliptin/placebo on symptoms and cognitive function tests.

Non-insulin adjunct therapy with Saxagliptin in c-peptide negative type 1 diabetes had no effect on symptom and cognitive responses to acute hypoglycaemia. (a.)Total Hypoglycaemia Symptom Score during euglycemic and hypoglycaemic plateaus. (b) Trail making B score (c) Digit symbol substitution score. Saxagliptin group shown by black bars, Placebo by white bars. Values shown as Mean ±SEM.

Discussion

It has recently come to light that there is dysregulation of glucagon release in patients with T1D alongside destruction of pancreatic β cells. This leads to a paradoxical increase in glucagon in the post-prandial state, and a failure to secrete glucagon during hypoglycaemia (Gerich, Langlois et al. 1973, Dupre 2005).

The importance of glucagon's contribution to the hyperglycemic state in diabetes was elegantly demonstrated in a recent study of transgenic mice lacking functional glucagon receptors. When these mice were injected with streptozotocin to chemically destroy the majority of β -cells, overt diabetes did not develop despite marked insulinopenia (Lee, Wang et al. 2011). Studies such as these have led to the hypothesis that non-insulin adjunct therapy aimed at restoring, at least in part, physiological glucagon secretion would improve glycemic control in T1D. However, before such therapy can be advocated more widely, properly controlled, randomized clinical trials need to be performed in individuals with T1D to show improvement in acceptable outcome measures.

We sought to determine whether adjunct therapy with oral DPP-4 i, agents known to suppress glucagon secretion during hyper- and not hypoglycaemia, would improve glycemic control and or act as insulin sparing agents in T1D. In addition, we reasoned that some restoration of physiological glucagon secretion would reduce daily insulin requirements and therefore lead to improved (reduced) glucose variability and less frequent hypoglycaemia, potentially improving hypoglycaemia awareness and the counter-regulatory responses to hypoglycaemia. In this context, even if DPP-4 i did not improve overall glycemic control they might provide a safer means of achieving glycemic targets.

However, we report that 12-weeks of adjunct saxagliptin therapy in subjects with established T1D failed to show any significant benefit on glycemic (frequency of biochemical

hypoglycaemia, insulin doses, GV and HbA1c) or metabolic parameters (weight), and did not significantly improve either subjective awareness of hypoglycaemia (Gold score) or formal testing of counterregulatory responses by the hyperinsulinemic clamp.

The main limitations of the study include the size of the study cohort; while our study was adequately powered to detect a significant effect of DPP-4 i on adrenaline responses to hypoglycaemia using the hyperinsulinemic clamp, we are more limited in the conclusions we can reliably draw from our secondary analysis. In addition, assessment of glucose variability and hypoglycaemia frequency was made during periods of CGM over the 6 days of measurement and longer periods of assessment may have been more representative. However the robust methodology involved in the clamp studies is very suggestive that hypoglycaemia frequency was not reduced. Finally, we were not able to measure GLP-1, glucagon and c-peptide responses to a standard meal in the current trial so we cannot say for certain that saxagliptin therapy in T1D was effective at improving post-prandial glucose and glucagon responses. However, other DPP-4 i drugs in T1D have been shown to act in this way (Farngren, Persson et al. 2012), while in T2D saxagliptin significantly increases insulin secretion and improves glucagon area under the curve in the postprandial state (Henry, Smith et al. 2011, Sjostrand, Iqbal et al. 2014).

Our findings are consistent with a recent double-blind, randomized, parallel group study of DPP-4 i in T1D, which revealed that sitagliptin had no independent effect on HbA1c, insulin dose, weight, or C-peptide after 16 weeks of treatment. It was however noted that C-peptide positive patients randomized to sitagliptin had a non-significant trend toward decrease in HbA1c, mean glucose, and time spent in hyperglycaemia (Garg, Moser et al. 2013). It has been noted in trials so far, with both DPP-4 i and GLP-1 receptor agonists, that subjects who are c-peptide positive have a greater effect on post prandial glucose excursion and glucagon than those who are c-peptide negative (Creutzfeldt, Kleine et al. 1996, Dupre 2005)(as in our study),

and it has been suggested that not only is α cell suppression important in these patients but also the effect of delayed gastric emptying with these agents.

However, our results contrast with those of Ellis et al. (Ellis, Moser et al. 2011) who compared the DPP-4 i sitagliptin with placebo in a 4-week double-blind cross-over trial in 20 subjects with T1D. They reported that sitagliptin adjunct therapy significantly improved HbA1c (-2.91 ± 1.16 mmol/l) and glucose variability as assessed by M100, Glycemic Risk Assessment Diabetes Equation and J-index. However, this was a trial with a short duration of treatment and no wash-out period. There were apparent differences in baseline HbA1c between treatment arms and a marked Hawthorne effect suggesting that increased contact with health care personal and more frequent monitoring played a large part in the improvements seen. In addition, there were no significant changes in measures of GV (LBGI, MAGE, CV, SD) and even a trend to more time spent in the hypoglycaemia range (<3.5 mmol/l) in the sitagliptin group (Ellis, Moser et al. 2011).

The lack of effect of saxagliptin in the current study on HbA1c, daily insulin dose, and measures of GV suggests that oral DPP-4 i may have limited impact on basal or post-prandial glucagon in T1D. Although not specifically addressed in the current study through measurement of GLP-1, glucagon and c-peptide responses to a standard meal, in T2D saxagliptin has been shown to significantly increase insulin secretion and reduce glucagon area under the curve in the postprandial state (Henry, Smith et al. 2011, Sjostrand, Iqbal et al. 2014). In a study of 28 subjects with c-peptide negative and positive T1D, Farngren et al. (Farngren, Persson et al. 2012) reported that 28 days prior therapy with the DPP-4 i Vildagliptin significantly suppressed blood glucose and glucagon (approx. 9% reduction in AUC glucagon) responses to a standard breakfast meal test and increased basal and meal-stimulated GLP-1. This group also reported a small benefit of DPP-4 i in terms of HbA1c (Farngren, Persson et al. 2012). It is possible however that the effect of Vildagliptin in this study was indirect,

occurring via an enhancement of residual endogenous insulin secretion in those subjects who were c-peptide positive. This would be consistent with the findings of Garg et al. (Garg, Moser et al. 2013) who also found that the DPP-4 i sitagliptin in T1D increased post-meal GLP-1 and suppressed glucagon, and suggested the benefits of DPP-4 i tended to be in C-peptide positive patients.

DPP-4 i have the advantage of oral administration but the injectable therapies such as GLP-1 receptor agonists (Kielgast, Holst et al. 2011) or amylin analogues (Chase, Lutz et al. 2009), may have a more potent effect on the α cell, and hence be a more suitable adjunct therapy. Overall, more convincing trials need to be reported before the case of α cell directed therapies can be implemented.

In summary, the present study demonstrates that in subjects with moderately well-controlled C peptide –ve long-standing T1D, non-insulin adjunct therapy with the DPP-4 i saxagliptin has no measureable effect on HbA1c, glucose-variability or symptomatic awareness and counterregulatory responses to acute hypoglycaemia. These findings do not support the use of DPP-4 i in the management of T1D.

CHAPTER 6;

STUDY 3; THE GV COHORT; Glycaemic variability and the cortisol curve

Introduction

The impact of GV in T1D is highly debated. Clearly, there is a strong relationship between GV and hypoglycaemia. Glucose variability has been shown to independently predict severe hypoglycaemia risk in the DCCT (1995) (Kovatchev, Cox et al. 2000). In this context, hypoglycaemia recently emerged as a significant risk factor for CVD mortality in 3 multi-center intervention trials in Type 2 diabetes (Gerstein, Miller et al. 2008, Patel, MacMahon et al. 2008, Duckworth, Abraira et al. 2009)

The association between GV and hypoglycaemia is the basis of STUDY 2, but we now wanted to explore additional potential effects of GV and in particular recurrent hypoglycaemia (RH) on whole body physiology. It has recently been proposed that GV, a measure of the fluctuation of glucose reading around the mean daily value, may be involved in the pathogenesis of macrovascular disease. The Verona Diabetes Study showed glycaemic variation was a significant and independent predictor of mortality. (Muggeo, Zoppini et al. 2000). The mechanisms through which glycaemic variability might accelerate progression of macrovascular disease are unclear although increased glucose variability has a negative impact on cell apoptosis and oxidative stress (Li, Liu et al. 1996, Quagliaro, Piconi et al. 2003). Monnier et al demonstrated induced glycaemic variability caused a dramatic fall in prostacyclin synthetase, a major anti-atherogenic endothelial cell enzyme. Glucose fluctuations have also been shown to have harmful effects on retinal and kidney vasculature. (Li, Liu et al. 1996, Jones, Saunders et al. 1999)

While hyperglycaemia is clearly implicated in the development and progression of microvascular complications in T1D, the association between hyperglycaemia and macrovascular complications is not clear, and therapeutic interventions targeting glucose control have so far failed to show a marked reduction in adverse cardiovascular outcomes. Certainly, T1D is associated with an increase in all-cause and cardiovascular (CVD) mortality. Data from the UK General Practice Research Database revealed the hazard ratio for major CVD was 3.6 (95%CI 2.9-4.5) in Type 1 diabetic men and 7.6 (5.5 – 10.7) in women (Soedamah-Muthu, Fuller et al. 2006). A recent prospective cohort study from the Scottish Diabetes Research Network epidemiology group reported that the estimated loss in life expectancy is still related to ischemic heart disease (36% in men, 31% in women (Livingstone, Levin et al. 2015).

Hypoglycaemia however presents a profound stimulation to the HPA axis and stimulates a rise in peripheral glucocorticoid, while repeated hypoglycaemia may eventually lead to a down-regulation of this response. This represents a form of ‘stress habituation’, which has been shown to lead to blunting of cortisol diurnal variation (Heim, Ehlert et al. 2000). In the Whitehall II study, blunting of the diurnal cortisol curve was linked to increased risk of all-cause mortality (Kumari, Shipley et al. 2011). Diurnal cortisol decline has also been related to increased coronary calcification in the CARDIA study. (Matthews, Schwartz et al. 2006).

Thus, increased glucose variability and repeated hypoglycaemia may, independently from chronic hyperglycaemia, contribute to CVD mortality in T1DM. In STUDY 3, we explored whether glucose variability and/or repeated hypoglycaemia adversely affects the daily diurnal cortisol profile. If this is the case, we hypothesize could this play a contributing factor to the excess cardiovascular events seen in T1D?

Methods

This study was designed to focus on the effects of both recurrent hypoglycaemia and glycaemic variability on the HPA axis.

Following informed consent, a structured interview enabled collection of demographic details, diabetes and hypoglycaemia history. Following this the subject was fitted with a blinded professional CGM device (iPRO) and given a calibration diary (to record blood glucose readings at least 4 times a day as detailed by Medtronic). Subjects were encouraged to wear the monitor for 7 days, but shorter periods (min 4 days) due to other commitments would be possible. Subjects were given 8 pre-labelled sample bottles to collect the saliva (for measurement of cortisol) at pre-determined times –see Appendix 1. Subjects were advised to collect the sample, not more than 3 days prior to returning it back to myself. Samples were stored at room temperature until it was returned back. On receipt of the samples, the samples were stored at -20°C until analysis.

During the 2nd visit, the subject's iPRO device was downloaded with the aid of the calibration readings. The raw glucose values were exported to the EasyGV version 8.7 program (an excel sheet with pre-determined formulae, which enables calculation of several GV parameters from the raw glucose data).

During the analysis phase, subjects with T1D were divided into those with low exposure to hypoglycaemia (LBGI<5) and those with high exposure to hypoglycaemia (LBGI>5).

Age, sex and BMI matched non-diabetic control subjects were also recruited to match these 2 groups with diabetes.

Assessment of GV - Continuous glucose monitoring

Most of the studies to date have examined the impact of GV on diabetes-related complications by further analysing the DCCT/EDIC database and have primarily used SMBG as a means of assessing glucose variability. SMBG (usually 7 point) has many limitations including patient reliability in taking adequate readings, and the absence of data from the nocturnal period. Current continuous glucose monitoring (CGM) systems overcome both these problems, and are capable of capturing data at 5 minute intervals for a duration of 5-7 days. They are able to therefore provide a much more robust means of measuring short term glucose variability. It is particularly valuable during times when SHBG cannot be performed (i.e. during sleeping).

In our study, the sterile disposable continuous glucose monitoring (CGM) sensor iPro CGM system (Medtronic, Northridge, CA) was inserted subcutaneously. Subjects were instructed to record at least 4 glucometer readings (preferably pre-meal and bedtime) for calibration purposes. CGM was worn for an average of 5-7 days. Data was recorded every 5 minutes. At the end of the monitoring period, the sensor and meter data were downloaded on the Carelink iPRO Therapy Management Software for diabetes. The data was then transferred to the Easy GV software, where, various metrics of glucose variability were calculated. We considered Low Blood Glucose Index (LBGI)/High Blood Glucose Index (HBGI) as an indicator of hypo and hyperglycaemic stress. Average Daily Risk Ratio (ADRR) was also calculated to take into account both extremes. Other measures indicative of short lived glucose variability were assessed by measuring SD, MAGE and CONGA.

Accuracy

CGM systems are often assessed for accuracy through evaluation of MARD (Mean Absolute Relative Difference). This is calculated by comparing the mean of the duplicate capillary BG readings which are then paired to interpolate CGM readings. The difference in the 2 readings is divided by the mean of the duplicate capillary BG measurement results. The Enlite sensor (using the Veo calibration algorithm) produced a MARD of 13.9% with 97.3% of points within A+B zones of the Clarke error grid (Keenan, Mastrototaro et al. 2012).

The SG:BG (sensor glucose: blood glucose), which is % of readings within 1.1mmol/L of capillary readings has been reported as 88.7% (2.2-4.4mmol/L), 83.3% (4.4-6.6), 94.9%(13.3-22.2) for the sof-sensor(Welsh, Kaufman et al. 2012) which was used in this study.

Time lag

There have been many criticisms regarding the time lag in continuous glucose monitoring systems. However a recent study using isotope tracer technology and micro dialysis techniques sampling from the interstium, has shown that in the fasting state, the physiological delay from the vascular compartment to the interstitium is actually only 5-6 minutes.(Basu, Dube et al. 2013)

Problems at hypoglycaemia range

Based on corresponding SMBG and CGMS, an abstract presented at the ADA(choudhary 2006) suggested a cut off value for detection of hypoglycaemia of 3.3mmol/mol. based on the optimal sensitivity and specificity (0.73 and 0.73 respectively) at this level, and showed that at lower levels, although there is an increase in sensitivity, this is at the expense of lower specificity (0.92 and 0.38).

Assessment of HPA axis

95% of secreted cortisol is bound to large proteins such as Cortisol Binding Globulin and albumin. Due to its low molecular weight and lipophilic nature, cortisol moves into cells by passive diffusion, and therefore it is possible to measure cortisol in all bodily fluids. Most of the cortisol eventually binds to either the cytosolic mineralocorticoid or glucocorticoid receptors, and it is only the unbound fraction which is thought to be biologically active.

Saliva

It has been 30 years since steroids were first looked at in the saliva. Saliva is mostly produced by three pairs of glands (parotid, submandibular and sublingual), with variable amounts of crevicular fluid from the tooth-gum margin, as well as exudates from plasma or blood from abrasions or other lesions. Neutral hormones generally enter by rapid diffusion through acinar cells and more importantly is independent of rate of saliva flow. (Vining, McGinley et al. 1983). There is poor correlation between total cortisol levels in plasma and saliva, because of variable amounts of CBG. Several studies have shown a good correlation between paired saliva samples and plasma cortisol, however correlation has been found to be best when cortisol levels are less than 500nmol/L (Putignano, Dubini et al. 2001) which is approximate saturation point of CBG binding affinity.

However a number of studies have shown that unbound cortisol (which is not affected by CBG) either in serum or plasma samples correlates very well to salivary samples ($r > 0.90$). However due to the high concentration of 11β hydroxysteroid dehydrogenase in the saliva, part of the cortisol is converted to cortisone, therefore the salivary cortisol represents only about 60-100% of the free fraction in the circulation, and is about 4-6% of the total plasma concentration.

Single basal cortisol (unstimulated) has been used in many studies but is a poor indicator of the HPA axis, due to the low intra-individual stability (Coste, Strauch et al. 1994) and large inter-individual variation. However day time cortisol profile have been shown to be a much better indicator of HPA activity. In contrast, urinary measurement of cortisol shows an inability to assess rapid changes in cortisol levels.

All subjects were provided with verbal and written instructions for collecting saliva samples using the simple tongue and cheek sucking, following which they would spit out into a wide topped universal container. Subjects were asked to collect 7 saliva samples throughout a chosen day (at wakening, +30mins, +45mins after wakening, +3, 6, 9 and 12 hours after wakening). They were advised to wake up promptly, to get an accurate time of wakening, to avoid food consumption and brushing teeth for at least 30 minutes before collection and to refrain from exercise and alcohol both on the day of collection and 24 hours prior. They were advised to rinse their mouth just prior to collection and to avoid contamination with debris. All blood stained samples were discarded, and subjects were advised to repeat the sample. Subjects were advised to collect the sample, not more than 3 days prior to returning it back to the research team. Samples were stored at room temperature until it was returned back. On receipt of the samples, the samples were stored at -20°C until analysis.

Advantages

The main advantages are that salivary assessment is non-invasive, stress free and real time, and is possible done during a subjects' normal day, rather than serum samples which would require extended stay at the healthcare setting or multiple repeated blood sampling. Salivary sampling also measures free unbound hormone (biologically active) and is therefore not confounded by high affinity binding proteins (Vining and McGinley 1987). Salivary cortisol from the earliest reports have shown to be a good reflection of overall unbound levels in the

plasma, and particularly it has been shown to reflect the diurnal variation seen in plasma cortisol. (James, Walker et al. 2004) Salivary cortisol has also been found to be stable across widely varying temperatures and movement, as was demonstrated in a study which compared levels of cortisol between saliva which had been frozen within 1 hour and saliva which had been exposed to different temperatures over 5 days($R^2=0.92$, $p<0.001$) (Clements and Parker 1998). It has been further confirmed that the enzyme immunoassay method can be used reliably when measuring cortisol levels when the samples have been exposed to multiple different temperatures (-18 to room temperature for up to 72 hours) and therefore home sampling has been shown to be a reliable way of collecting real life samples(Nalla, Thomsen et al. 2015)

Disadvantages

Measurement of cortisol can be challenging as it can be affected by several external factors. Amongst others acute stress cannot be controlled during these collection periods. Other factors which could influence levels would be differences during working days and weekends, variations in waking time and sleep duration prior to collection.

Measurement of cortisol requires dealing with the difficult matrix of saliva, and this is often through multiple freeze-thaw cycles and centrifugation. Steroids are also present at a much lower concentration in the saliva, which again can lead to problems with analysis. Blood contamination has been shown to be associated with presence of both sex hormone binding globulin and corticosteroid binding globulin (CBG), which may interfere with measurement of unbound hormone. (Hammond and Langley 1986) Saliva cortisol is not a good marker of total plasma cortisol because of the presence of CBG which is saturated up to a level of 500-600nmol/L of cortisol, however it correlates better with free cortisol. The salivary gland has abundant 11-Beta hydroxysteroid dehydrogenase type 2 activity and therefore saliva has

up to 3 times cortisone level as opposed to plasma (10% of cortisol level), therefore the assay- cortisol antibodies has to be found not to interfere with cortisone, otherwise there could be quite variable levels. (Lewis 2006)

Various pitfalls in collection of saliva has been seen with stimulated saliva(Gordon, Peloso et al. 2005) (that is through chewing), with both increased variability and alteration of true levels.(Vialard-Miguel, Belaidi et al. 2005), and therefore experts (Lewis 2006) have suggested unstimulated saliva is often best.

Methodological issues

a. It relies heavily on proper adherence to the sampling protocol, particularly the early morning rise is heavily reliant on the first sample being done on waking, and subsequent samples done within the first 45 minutes of waking. A study (n=47) using an electronic monitoring advice showed that only 74% were found to be compliant with the salivary collection schedule. The diurnal rhythm was significantly different for the compliant and non-compliant groups. Most importantly, increased compliance was associated with a good rise of cortisol on wakening and the steepness of the cortisol decline during the latter part of the day. 49% of the group were also informed of the nature of the electronic device, and had greater adherence to the protocol, compared to the non-informed group(Kudielka, Broderick et al. 2003).

b. It is important that food or drink intake is avoided for 1-2 hours prior to sampling, due to variations in salivary secretion which in turn will negatively affect the result.

Particularly foods that lower the mouth pH, can compromise antibody-antigen binding and enzyme activity thus leading to invalid immunoassay results(Papacosta and Nassis 2011).

This may not always be possible in subjects experiencing recurrent hypoglycaemia, where prompt treatment is essential.

- c. Alcohol consumption 24 hour prior to sampling is known to increase saliva secretion and may also influence the results(Martin and Pangborn 1971). Alcohol consumed the night before salivary sampling has also been shown to decrease CAR the following day(Stalder, Hucklebridge et al. 2009). For this reason, we instructed all participants to avoid alcohol for at least 24 hours prior to salivary sampling. Chronic consumption of alcohol has also been shown to increase CAR particularly in women(Badrack, Bobak et al. 2008) and also blunt the cortisol decline over the day, suggesting alcohol consumption activates the HPA axis.
- d. A further confounder was smoking. Smoking acutely increases cortisol levels in a dose dependent manner (Rohleder and Kirschbaum 2006). Chronic exposure to smoking has also shown to cause higher basal cortisol levels and greater morning rises and a lesser decline in cortisol over the day(Kumari, Badrick et al. 2010, Direk, Newson et al. 2011). Therefore smokers were excluded.
- e. Women who were on oestrogen therapy were excluded. This was done as rodent studies have shown that oestrogen treatment was able to normalise any alteration of the HPA axis such as altered response to stress and an ineffective steroid negative feedback mechanism.
- f. Blood contamination of saliva through abrasions in the oral cavity can also significantly affect the results, as plasma cortisol is typically 200-800nmol/L but in saliva is only 3-30nmol/L, and so if there is blood leakage, it can increase the concentration of salivary cortisol levels. This can be determined indirectly, by ensuring that albumin levels in the saliva do not exceed the threshold levels(Papacosta and Nassis 2011).
- g. Multiple days of sampling may be better to assess the HPA axis, however this is not feasible in many studies. (Hellhammer, Fries et al. 2007). One off assessment of HPA using cortisol samples spread out over a day, only gives us a cross sectional view of the data.

Recruitment

Currently there is no literature looking at levels of salivary cortisol and diurnal patterns in a population with T1D. Therefore, we have calculated our sample size using a population study looking at salivary cortisol in a middle aged healthy community in Germany (Lederbogen, Kuhner et al. 2010) (Chen, Couto et al. 2006). Using this, the AUC of the salivary cortisol diurnal curve was roughly 100 in a healthy population. To determine a 25% change in the AUC of the cortisol curve, between the low and high glucose variability, with a power of 80%, we needed 49 patients in each group. We aim to recruit 75 participants in each group to complete 49 in each group to account for incorrect sampling or patients not returning salivary cortisol samples.

Approvals were sought from our local sponsorship committee (Ref; 2012DM05) (Joint sponsorship from University of Dundee/NHS Tayside), local ethics committee (East of Scotland Research Ethics Service (EoSRES) REC 2) REC ref; 12/ES/0048 and also from the local Research and Development (R&D) office at Tayside.

Subjects were identified through one of the recruitment strategies outlined below.

1. Participants were initially approached either by PI (PG) or Research Nurse. This was usually a visit organized through the NHS, whereby patients are given the opportunity to have a period of continuous glucose monitoring. Patients will be given a patient information sheet at this point to consider the research information. Patients will return their CGM a few days later, and at this point, they will be asked whether they would be willing to take part in the trial. Following informed consent, screening of the patient will take place. If the patient is screen +ve, then they will be asked all the other questions pertinent to the study and will be given the salivary cortisol containers

with instructions, and a pre-paid envelope to return the containers. An agreement is in place with the clinic consultants with regard to this search strategy.

2. Participants were identified through the SDRN (Scottish Diabetes Research Network), who fit the inclusion and exclusion criteria. The research team have an agreement with SDRN to perform these searches. These patients have agreed to be contacted. Patients would be contacted by phone to see if they would like to be involved in this research. A study letter and patient information sheet (PIS) will be sent by the PI to the participants. Participants will be contacted by phone by member of the research team (>24 hours) to arrange an appointment to discuss the study, give consent and undergo screening.
3. Suitable participants attending for routine diabetic clinic appointments within NHS Tayside will be approached by a member of the research team (PI, research nurse) and asked if they would consider taking part. If they agree, a PIS was provided. Participants will then be contacted at least 24 hours later, to ask if they would like to be involved in the study. If so they will be invited for screening visit and fitted with a continuous glucose monitor, and given the salivary cortisol containers and instructions. A verbal agreement is in place with clinic physicians to approach patients in this way.
4. Participants were identified through the SCI-DC Diabetes Clinical Database who fit the inclusion/exclusion criteria. Caldicott approval has been sought to enable the research team to screen this database for potential participants. We will write to the patients on behalf of the NHS Consultant, to see if they would like to take part. The PIS that the patients are sent will have a tear off slip/email to enable participants to agree to be contacted. The research team will then contact them.

Control –Healthy, non-diabetics

5. Partners of participants if agreeable will be contacted after consent from the Participant. The participant will be given a participant information sheet to be given to their partner, with a tear off slip/email which if there is a reply, then the partner will be contacted.
6. Participants will be recruited from advertising on the University Website.
7. Participants will be recruited from posters that will be placed around Ninewells Hospital and Medical School.

Inclusion criteria

1. Group 1
 - a. T1D
 - b. Age 18-60
 - c. Low glucose variability (LBGI<5)
 - d. Ability to give written consent to participate in the study
2. Group 2
 - a. T1D
 - b. Age 18-60
 - c. High glucose variability (LBGI≥5)
 - d. Ability to give written consent to participate in the study
3. Group 3
 - a. Non-diabetic
 - b. Age 18-60 years
 - c. Ability to give written consent to participate in the study

Exclusion criteria

1. Acute physical or mental illness
2. Those on steroid treatment
3. Those who would not follow salivary protocol (due to inability to stop smoking on the day of sampling, or unable not to have alcohol on the day of sampling)
4. Those who have Cushing's syndrome
5. Those who had primary or secondary adrenocortical failure
6. Pregnancy or on greater than 36 mcg oestrogen treatment (alters circulating levels of Cortisol Binding Globulin)
7. Breastfeeding mothers
8. If in compliance with sampling procedure of salivary cortisol or if it exceeded 7 days before salivary collection and sample arrival. (At least 4 out of the 7 timed samples need to be done at the time windows for valid analysis)

Study visits

1st visit; Screening visit

Informed consent obtained

History

Eligibility criteria checked

Demographics obtained (BMI, Gender, Ethnicity, Marital status, Education, Occupational status, Physical activity)

Diabetes history (Duration, HbA1c, insulin doses, frequency of hypos, evidence of microvascular and macrovascular complications)

General medical history (Past medical and psychiatric history, SF-12 and PHQ-9)

Smoking and alcohol history

Diabetes Specific Questionnaires (Gold questionnaire, Composite score, Hypoglycaemia Fear survey –II, High Blood Sugar survey)

Continuous Glucose Monitor (CGM) insertion

Medtronic I-PRO device will be inserted in an appropriate place (usual site will be in the abdomen). Subjects will be advised to keep a detailed food and exercise diary. They will also be asked to enter blood sugar readings at least twice a day, usually before each meal for calibration purposes. They will be advised to enter insulin doses and carb content in the monitoring sheets provided.

Sample collection

Subjects will be given the salivary log sheets (see appendix) and 7 labelled containers to collect saliva as per the collection instructions (see appendix). This will be done whilst the subject is wearing the CGM, so that glycaemic variability can be correlated with the cortisol diurnal patterns.

2nd visit

Subjects will be seen 4-7 days after insertion of the CGM. The PI will go through the CGM in detail and provide advice (insulin doses, carb counting, if not carb counting advice and basic information will also be provided, tailoring therapy for exercise, advice regarding hypoglycaemia treatment and prevention). This part of the exercise is not directly related to this PhD but will be provided as a service to the participants.

Subjects will also return the salivary samples with the log. This will be stored in -20 Deg C freezer, until analysis.

Visit 2/3

The last 30 subjects of this cohort of participants will also have a CRH (Corticotrophin Releasing Hormone) stimulation study. Participants will have 1 cannulae inserted in the ante-takes place. Participants will have the CRH (1mcg/kg) injected intravenously over 1 minute. All studies will be performed after 5pm to allow the basal cortisol levels to be at its lowest. Two baseline blood tests will be done (-15 and -5 minutes before the CRH injection). Following CRH injection, blood samples will be taken every 15 minutes for the first hour and then every 30 minutes for the second hour. (8 samples in total). These will be analysed for ACTH, cortisol and adrenaline.

Results

In total, 178 subjects were screened for suitability. 28 subjects were excluded. 12 were excluded because of smoking status, 4 were excluded because of oestrogen therapy, 1 had an ongoing physical illness, 1 had significant psychiatric problems, 8 withdrew consent, 2 were on steroid therapy. Finally we had 50 subjects with low LBG1 (less than 5), and 50 subjects with high LBG1 (≥ 5) and 50 non-diabetic controls.

Table 10; Baseline characteristics of the original cortisol cohort. N=50 in each group.

| | Low LBG1 | High LBG1 | Control |
|---|------------|------------|------------|
| Age (years) | 42.1 (1.7) | 40.7 (1.8) | 39.6 (1.5) |
| % male | 52 | 38 | 40 |
| BMI (% of total group)kg/m ² | 44 | 36 | 36 |
| 18-24.9 | | | |
| 25-29.9 | 38 | 52 | 42 |
| >30 | 18 | 12 | 22 |

| | | | |
|--|-------------|-------------|-----------|
| Duration of diabetes (years) | 20.9 (11.5) | 23.7 (10.8) | |
| HbA1c (%) | 8.9 (1.4) | 9.1 (1.7) | |
| Basal/bolus split (units) | 47.4/52.6 | 51.5/48.5 | |
| Insulin units/kg | 0.7 (0.2) | 0.7 (0.4) | |
| Complications | | | |
| Retinopathy (% of total group) | 30 | 20 | |
| Nephropathy (% with micro/macroalbuminuria) | 10 | 14 | |
| Neuropathy (% with any evidence) | 20 | 26 | |
| Marital status (% married/living with someone) | 72 | 86 | 62 |
| Alcohol (units/week) | 6.7 (1.3) | 4 (1) | 4.6 (0.7) |
| Years in education | 14.6 (0.5) | 14.8 (0.4) | 17 (0.5) |
| In employment/student (% of whole group) | 82 | 80 | 96 |
| High job strain (% of whole group) | 54 | 48 | 44 |
| Good Social support (% of whole group) | 96 | 90 | 90 |
| Physical activity (% of whole group) | 26 | 42 | 58 |

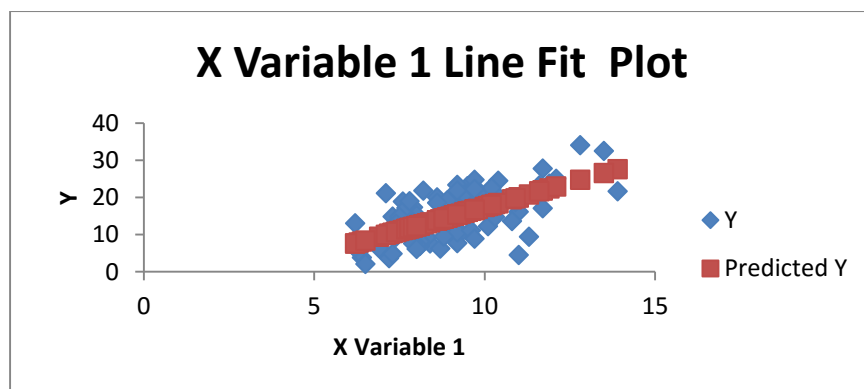
Reliability of this data set

1. Frequency of reported SH was shown to predict computed LBGI using 4-7 days of CGM data

Subjects were asked to report frequency of severe hypoglycaemia (SH) over the last 2 years. Before the subject volunteered this information, the definition of SH was explained to them.

SH was defined for the purpose of this study, to be all episodes which required third party assistance because of incapability of the subject. The frequency of SH significantly predicted the LBGI computed from the CGM data set. ($R^2=0.06$; $F=4.74$; $df=1$; $p=0.03$) It is therefore suggested from this data set, that previous SH, resulted in higher LBGI during this period of CGM monitoring. I feel this gives this dataset (captured over 5-7 days), some backing that it is reflective of a subjects usual hypoglycaemic exposure

2. HbA1c was found to significantly predict HBGI



HbA1c measures were found to significantly predict the HBGI computed from the 4-7 days of CGM. ($R^2=0.39$; $F=59.6$; $df=1$; $p<0.05$). This again is in keeping with published data.

In summary, both these validity checks suggest that the period of CGM monitoring was reflective of both their previous exposure to hypo and hyperglycaemia.

When the data points were examined for normality using the D'Agostino & Pearson omnibus normality test and also the Shapiro-Wilk normality test, it was ascertained that the data was not normally distributed with each time point measured. Therefore all data underwent square-root transformation, which normalised the data set. All summary measures were calculated using the transformed data.

Cleaning data

Due to the nature of salivary sampling, in obtaining repeated measures over the course of one day, there were subjects who had not given us saliva for all 7 time points during the course of one day. Other reasons for missing data, was poor repeatability during analysis of the cortisol levels, and very low levels detected. Means and standard deviations were calculated for each time point for each group (Low, High, and Control). If a result was out with of 2 SD, then that result was removed and thought to be spurious. A reason for this includes, if the subject had gum pathology that can spuriously, elevate the salivary cortisol levels. This would particularly be suspected if there were numerous high readings for one day on a particular individual.

Table 11; Individual data points which were excluded from the main data set.

***very low levels were often seen at the end of the day, and therefore due to technical reasons, could not be measured accurately.*

| Missing data | Low LBGI | High LBGI | Control |
|--|------------|-----------|-----------|
| Poor repeatability during analysis | 11 | 4 | 6 |
| Very low levels* | 6 | 3 | 3 |
| Forgotten sample | 7 | 8 | 13 |
| Result out with mean +2SD (outliers) | 7 | 18 | 12 |
| Total % of data points- missing | 33/350= 9% | 33/350=9% | 34/350=9% |

Area under the curve (AUC)

The AUC was calculated using Graphpad Prism. Prism computes the AUC by connecting points by straight lines and then utilising the trapezoidal method. It calculates the area of each trapezoid by calculating the area of the equivalent rectangle. The sum of all the rectangles would then give the AUC. It computes the figure from the first x value (first salivary sample of the day), till the last x value (this maybe 9 or 12 hours, depending on the last sample). If first value is not the wakening sample, prism will use the first x value (first time sample of the day), and calculate AUC from there. It is more likely, there will be bigger impact on AUC calculations, if first value is missing rather than last value. The numbers of first missing values, are equivocal in the 3 groups, and therefore should not make a major impact on the calculations.

Table 12 No of missing first values in each group

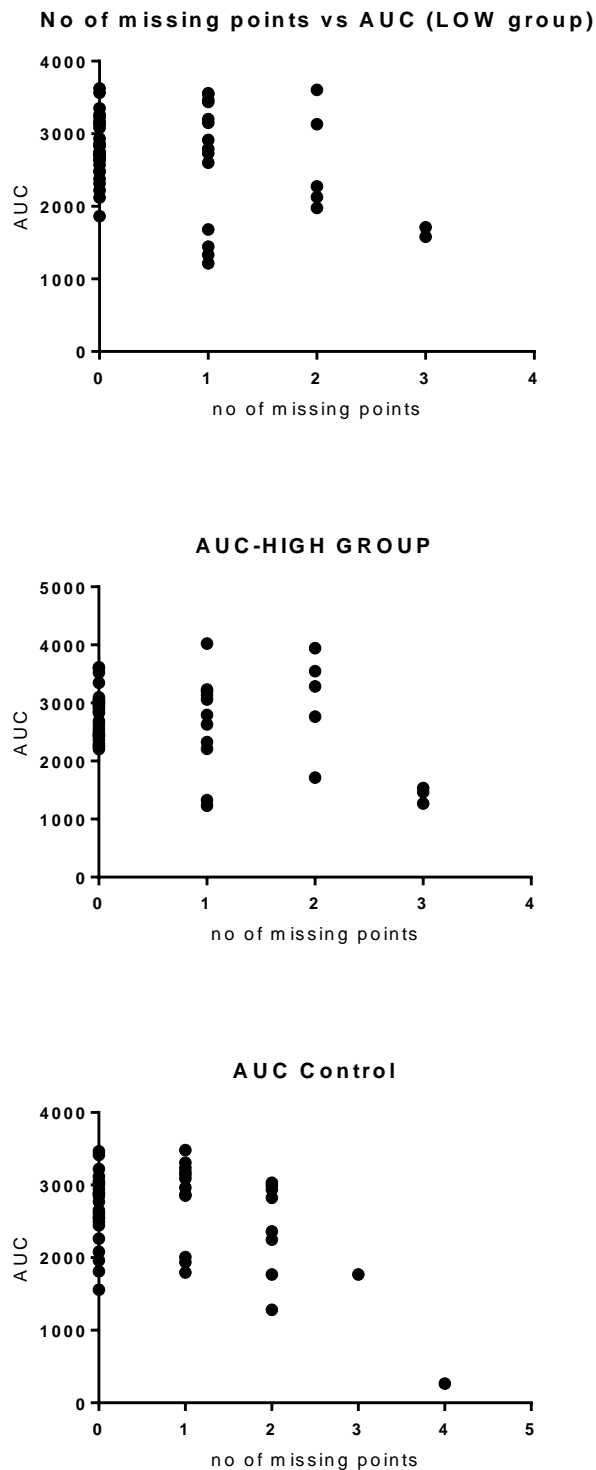
| Group | No of missing 1 st values |
|-----------|--------------------------------------|
| Low LBGI | 3 |
| High LBGI | 4 |
| Control | 3 |

I carried out an AUC analysis using all my data, and left the missing points as “blank” in the data sets. The graphs below show the impact of missing points on AUC calculations. It is clear that 3 and 4 time points missing in an individual clearly shift the AUC downwards, and therefore these subjects were removed from further analysis.

Table 13; No of subjects in each group with either 3 or 4 missing points

| | |
|-----------|---|
| Low LBGI | 2 |
| High LBGI | 3 |
| Control | 2 |

Figure 22; Graphs showing impact of missing points on a. low LBGI b. high LBGI c. control



As LBGI (Low Blood glucose index) has been shown to be predictive of severe hypoglycaemia, these 2 variables were compared in the raw data set, for any discrepancies

Table 14; Table correlating the no of subjects with severe hypoglycaemia and their LBG I scores.

| No of subjects | Low LBG I | High LBG I |
|-----------------|-----------|------------|
| Frequency of SH | 5 | 8 |
| 1 or 2 episodes | | |
| >5 | 1 | 4 |

One patient in the Low LBG I had more than 5 SH in the last 2 years. This was a patient, who was referred to the diabetes clinic from a peripheral centre, due to IAH and recurrent severe hypoglycaemia. On examination she had significant lipohypertrophy on the abdomen due to her insulin injections, and she was on a total of 120 units of insulin/day. Her insulin doses were reduced by 40% initially. This led to a dramatic reduction in her frequency of hypoglycaemia. Therefore her CGM which she had a few days later (after considering the PIS), did not reveal her prior exposure to hypoglycaemia. This subject was also removed from the analysis. Therefore the total number of subjects after removing the subjects with 3 or 4 missing values, and also removing the above subject was

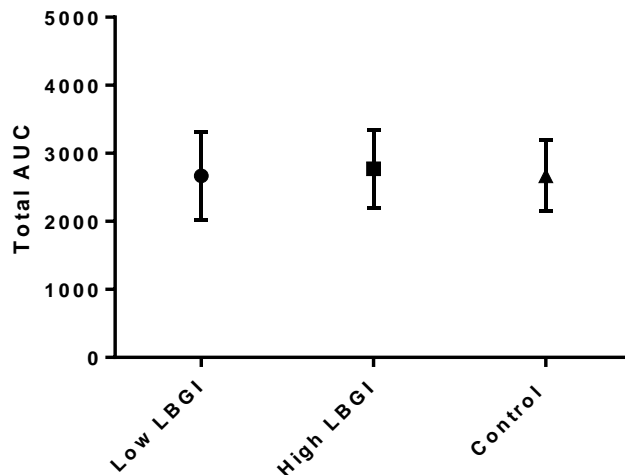
Table 15; Total number of subjects (excluding single pt with discrepancy between CGM and hypo frequency) analysed after removing those with 3 or 4 points

| | Low LBG I | High LBG I | Control |
|-----------------------------------|-------------------|------------|---------|
| No of subjects removed | 2 + 1 (above) = 3 | 3 | 2 |
| Total number of subjects analysed | 47 | 47 | 48 |

AUC was then calculated on these remaining subjects

The graph below shows the AUC for each of the 3 groups

Figure 23; Graphical representation of AUC of mean and confidence intervals for each of the groups



There was no significant difference for the total AUC for each of the 3 groups. ($F_{2,139}=0.518$ $p=0.5969$). Since both early morning cortisol levels (CAR) and the diurnal decline are under different regulatory control (as they have shown not to correlate), it has been suggested that AUC of these different periods need to be looked at separately. There was no significant difference in the wakening AUC cortisol (0-45mins) between the 3 groups ($\kappa_w 0.8714$). AUC was also calculated for the curve between (3 and 12 hours). However, 2 or more readings missing in these calculations, significantly impacted the AUC on visual inspection. Therefore these subjects were removed to avoid distortion of the data.

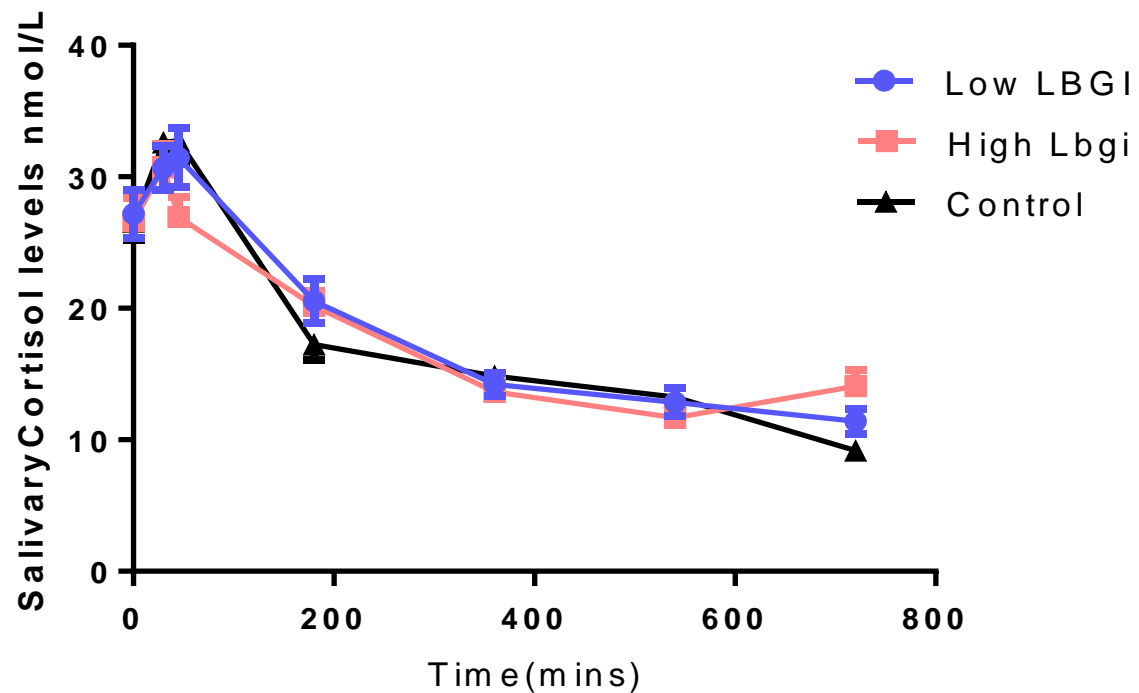
Table 16; Total number of subjects analysed after removing subjects with 2 or more individual readings missing

| | Low LBGI | High LBGI | Control |
|-----------------------------------|----------|-----------|---------|
| No of subjects removed | 1 | 3 | 0 |
| Total number of subjects analysed | 46 | 44 | 48 |

There was a significant difference between the AUC (3-12) groups $F(2,135)=3.081$ $p=0.0492$. This was driven by a significant increase in AUC between the High LBGI group and Control groups. (31.8 (0.9) vs 28.5 (1.1) $p=0.0239$).

Figure 24; Graph charting the salivary cortisol levels throughout the day.

Time 0 is time of awakening.



Mean levels

Mean levels were calculated for both the first 45 minutes and also for the 3-12 hour period, to determine if there was any evidence of hypercortisolaemia evident in either of these time periods. There was a significant increase in the mean levels of cortisol between the 3 groups, and this was only seen in the 3-12 hour period. This was primarily driven by a significant increase between both groups with diabetes and the control group, with no difference between the Low and High LBGI groups.

Table 17; Mean cortisol levels (standard error of the mean) of the 2 main periods of the day.

| | Low LBGI | High LBGI | Control | |
|---|-----------|-----------|-----------|-----------------------------|
| Mean (SEM) levels over the first 45 mins | 5.7(0.14) | 5.2(0.13) | 5.1(0.14) | F (2, 139) = 0.5 P = 0.5939 |
| Mean (SEM) levels over the 3-12 hour period | 3.7(0.11) | 3.7(0.10) | 3.4(0.11) | F (2, 139) = 3.2 P = 0.0456 |

A. First 45 minutes B. 3-12 hours after wakening. This shows the mean (standard error of the mean) cortisol levels divided in the 2 periods of the day (firstly the first 45 minutes of the day, then the mean levels over the next 3 to 12 hours after wakening).

Slope

Looking at the graph of the trend of cortisol levels through the day, it appears that the greatest decline occurs from the 45 min sample to the 3 hour sample, so this part of the curve was analysed first, particularly in relation to the downward slope.

This was calculated by subtracting the 3 hour cortisol value from the 45 min value, and then dividing it by the number of hours.

$$= (C_{45} - C_3) / 2.25$$

If a subject had a missing value in either the 45 min or the 3 hour samples, then they were excluded from this analysis

Table 18; Total number of subjects analysed after removing those subjects with Missing F45 (45 minutes after waking) or F3 (3 hours after waking) and also removing those subjects with 3 or 4 data points missing and also the one subject with the CGM which was not reflective of previous hypo exposure due to treatment regime change.

| | Low LBG1 | High LBG1 | Control |
|---|----------|-----------|---------|
| Missing F45 or F3 | 6 | 6 | 10 |
| Total number of subjects analysed out of the 50 | 41 | 41 | 38 |

Analysis of the slope from 45mins (peak period) to 3 hours after waking showed a mean (SD) slope of 0.48 (0.11) in the low LBG1 group vs 0.28 (0.09) in the high LBG1 group and 0.72 (0.09) units of cortisol decrease over time in the control group. There was a significant difference in the 3 groups for the slope. $F(2,117)=4.837$ $p=0.0096$. Univariate analysis showed that neither age $F(1,117)$ $p=0.188$ nor gender $F(1,117)$ $p=0.259$ did not have a significant influence on the slope.

I also looked at the slope for the whole day (from 45 mins to 12 hours after waking). If either of these values were missing, the next value (either later- for early samples or earlier- for last samples) was taken to calculate the slope.

Analysis of this slope which took into account the later samples revealed a similar pattern and showed a mean slope of 0.21 (0.02) in the lower LBG1 vs 0.14 (0.02) in the higher LBG1 vs 0.24 (0.02). There was a significant difference in the 3 groups for the daily slope ($F(2, 139)$ $=6.042$ $p=0.003$).

CAR

This looks at the dynamic of the initial rise in cortisol on waking. Several methods for calculating CAR have been described in the literature. These include mean increase (MnInc), area under the curve with respect to increase and ground. (AUC_i and AUC_g respectively) and mean cortisol levels. As both AUC_g and mean levels do not provide any information on the dynamics of the increase, investigators often use both a measure of the dynamics of increase (Mean increase or AUC_i) and a measurement of the value of the first sample.

Prior to running the analysis, those subjects with any missing values in the first 3 samples (F0, F30 and F45), were removed from the analysis, as there is currently no agreed method for imputation.

Table 19; Total number of subjects analysed after removal of subjects with missing points in F0, F30 and F45 (on waking, 30 minutes and 45 minutes after waking) and after removing those with 3 or 4 points missing + removing the 1 subject where CGM did not reflect previous hypo frequency, as insulin doses was reduced quite dramatically prior to insertion of CGM due to safety reasons.

| | Low LBGI | High LBGI | Control |
|------------------------------------|----------|-----------|---------|
| No of subjects with missing points | 9 | 5 | 9 |
| Total number of subjects analysed | 38 | 42 | 39 |

Thereafter, mean increase of cortisol rise was calculated as below

$$= (F45 + F30)/2 - F0$$

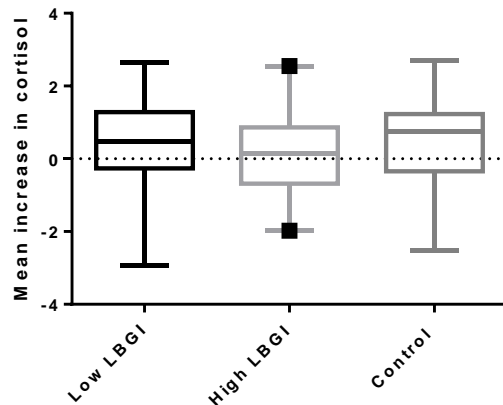
Mean increase for the 3 groups respectively (low LBGI, high LBGI, and Control) were

0.32(0.20) vs 0.16(0.17) vs 0.42(0.19). There was no significant difference between the CAR

in the 3 groups using this method. ($F_{2,139}=0.4128$ $p=0.5984$). Moreover, the distribution of the mean increase in CAR shows a large variation.

Figure 25; Box plot of mean increase in cortisol for each of the 3 groups.

Box extends from 25th to 75th percentile. Middle line is median. The whiskers are at 2.5 and 97.5 percentiles.



Box extends from 25th to 75th percentile. Middle line is median. The whiskers are at 2.5 and 97.5 percentiles.

This showed that 2 subjects were out with of 2.5 to 97.5 percentile. CAR was once again calculated with these outliers removed. Mean increase of CAR for the 3 groups were 0.31(0.20) vs 0.21(0.17) vs 0.42(0.19). Once again, there was no significant difference between the CAR between the 3 groups. $F(2,114)$ $p=0.74$.

A number of subjects did not show any rise in cortisol levels in the morning.

Table 20; Number of subjects analysed after removal of subjects with missing points within F0, F30 or F45 and removal of those where no CAR could be detected (no increase in cortisol levels from wakening)

| | Low LBG1 | High LBG1 | Control |
|---|----------|-----------|---------|
| No of subjects with Missing points within F0, F30 and F45 | 9 | 6 | 8 |
| No CAR detected | 8 | 13 | 5 |
| Total number now analysed | 30 | 29 | 34 |

The analysis was then carried out again by taking these subjects out of the analysis as well, to determine whether exclusion would have a significant impact on the analysis. This was done to exclude participants with a potential of incorrect sampling.

Mean CAR (using mean increase) for the 3 groups now was 0.81 (0.13) vs 0.63(0.18) vs 0.65(0.18). Once again, there was no significant difference between the 3 groups. (F (2, 90) $p=0.3368$. I also calculated AUC using the first 3 data points (F0, F30 and F45). If any of these values were missing, then the subjects were not included in this analysis.

Table 21; Total number of subjects used to analyse AUC_g by removing those subjects who had any missing values in the first 3 data points (F0, F30 and F45) and after removing those with 3 or 4 points missing totally + 1 subject where CGM did not reflect previous hypo frequency, as insulin doses were reduced quite dramatically prior to insertion of CGM due to safety reasons.

| | Low LBGI | High LBGI | Control |
|---|----------|-----------|---------|
| No of subjects with missing values in the wakening period | 9 | 6 | 8 |
| Total number of subjects analysed | 38 | 41 | 40 |

Mean AUC_g for the 3 groups (Low LBGI, High LBGI and Control) showed no significant difference (238.1 (5.8) vs 233 (6.6) vs 241 (5.9) F (2,116) p=0.41.

Mean AUC_i (AUC with respect to increase which takes into account dynamic increase of cortisol was calculated according to published calculations $(F30 + ((F45-F0)/2)) - 2 \cdot F0$. AUC_i showed no significant difference between groups F (2,115) p=0.95.

A two way ANOVA was conducted to examine the effect of gender and age on the CAR (mean increase). There was no statistical significant interaction between the effects of gender and age on the CAR. $F(3,84)=0.156$ p=0.93. Univariate analysis showed that age did not have a significant influence on the CAR. $F(28,25)$ p=0.58. I also performed linear regression, and this once again showed that age and gender did not statistically predict CAR (MnInc), $F(2,90)=0.45$ p=0.639.

Correlation analysis

Correlation coefficients between each of the glycaemic variability indices and the summary measures of the diurnal cortisol patterns are presented (see table 22)

The correlation between LBG1 and slope was inverse and statistically significant ($r_s = -0.23$; $p = 0.0393$) ie. so the greater the exposure to hypoglycaemia, the more blunted the slope. This was particularly the case in the early slope (calculated from 45 mins to 3 hours after wakening. (+ve slope indicates a downward slope, and a -ve slope indicates an upward slope)

Measures which are more indicative of higher glucose readings throughout the day (such as HBGI and mean) both show that there is a correlation between these measures and both Mean cortisol values of 3-12 hours and Total AUC. This correlation was inverse and statistically significant. So this data suggests that as mean glucose values and HBGI (which has been shown to be related to mean glucose) increase, the total AUC decreases.

| | Mean (0-45) | Mean (3-12) | CAR | CAR-AUC | Early slope (45-3 hours) | Late slope (3-12) | Total AUC |
|---------|-------------|---------------|---------------|----------|--------------------------|-------------------|---------------|
| LBGI | -0.040 | 0.083 | -0.027 | -0.036 | -0.23* | -0.15 | 0.12 |
| HBGI | 0.030 | -0.22* | -0.18 | 0.079 | 0.025 | -0.053 | -0.23* |
| ADRR | 0.043 | -0.15 | -0.16 | 0.073 | 0.086 | -0.0034 | -0.17 |
| Mean | 0.024 | -0.22* | -0.20 | 0.086 | 0.083 | -0.055 | -0.23* |
| SD | 0.0053 | -0.15 | -0.21 | 0.030 | -0.063 | -0.084 | -0.17 |
| LI | 0.026 | -0.077 | -0.29* | 0.0037 | -0.051 | -0.061 | -0.097 |
| CONGA | 0.015 | -0.23 | -0.19 | 0.083 | 0.058 | -0.066 | -0.24 |
| J index | 0.025 | -0.22* | -0.18 | 0.080 | 0.057 | -0.058 | -0.23* |
| MODD | -0.0093 | -0.17 | -0.13 | -0.00089 | 0.026 | 0.0080 | -0.17 |
| MAGE | 0.18 | -0.044 | -0.083 | 0.19 | 0.081 | -0.0075 | -0.10 |
| M-value | 0.045 | -0.22* | -0.18 | 0.083 | 0.037 | -0.050 | -0.21* |

Table 22; Correlation coefficients of the glycaemic variability indices to each of the main parameters of the cortisol curve. *denotes $p < 0.05$ and was statistically significant.

Discussion

The main finding of this ambulatory study was a significantly blunted slope of the cortisol curve in those with a high LBG (greater duration and depth of hypoglycaemia) compared to those with low LBG and the control (non-diabetic group). The blunting was significant between 45 minutes to 3 hour time interval ($p=0.01$, when typically in a healthy cohort, you would expect the fastest decline of cortisol. The blunting of the slope was also seen in the 2nd part of the slope (3-12 hours), with most significant blunting seen in those with high LBG ($p=0.003$). Many studies have showed that a blunted cortisol decline is associated with poor physical and psychosocial outcomes (Adam, Hawkley et al. 2006, Cohen, Schwartz et al. 2006).

This blunting of the cortisol decline was combined with a significantly increased AUC between 3-12 hours after wakening in those with a high LBG. This elevated cortisol in those with T1D, could be as a result of a number of mechanisms. This may be as a result of chronic hypersecretion of CRH due to recurrent exposure to hypoglycaemia. CRH may then cause a possible cortical adrenal hypertrophy. It appears that this effect of elevated cortisol in those in the high LBG group is over and above the effects of diabetes particularly when associated with poor glycaemic control. It has been shown in animal studies that poorly controlled diabetes also leads to an activated basal HPA axis, which consequently leads to increased baseline CRH and ACTH and total cortisol levels (Chan, Chan et al. 2001). In animals with STZ-induced diabetes, the HPA axis hyper activation appears to start at the level of CRH gene expression in the PVN, resulting in a strong feed forward cascade, with increased CRH, ACTH and corticosterone. However, some animal studies also report that insulin replacement results in partial resolution of this hyper-secretion state particularly of ACTH and glucocorticoid (Chan, Chan et al. 2002). Other effects are a decreased

hypothalamic pituitary feedback sensitivity and increased adrenal sensitivity to ACTH, with an associated adrenal hypertrophy (Revsin, van Wijk et al. 2008).

The elevated CRH has also been shown to stimulate the sympathoadrenal pathways, promoting release of adrenaline and noradrenaline, which actively promote glycogenolysis and gluconeogenesis further compounding to the glycaemic load (Clöre and Thurby-Hay 2009). However, in contrast, Davis and colleagues showed that a cortisol infusion resulted in a markedly reduced adrenaline response to IHH the following day, suggesting that it was the cortisol increase that was responsible for the defective counter-regulation (Davis, Shavers et al. 1996). This would fit with this data as those with high LBG (and more likely to have defective counter-regulation), showed the highest AUC (cortisol) with a blunted decline.

The adrenal gland has two distinct endocrine tissues; the steroid producing adrenocortical cells and the catecholamine producing chromaffin cells. The interaction between glucocorticosteroids and catecholamines is relatively poorly studied. Glucocorticosteroids induce the enzyme phenyl-N-methyl-transferase, a crucial enzyme involved with catecholamine synthesis (Cole, Blendy et al. 1995). Those with Addison's disease have been shown to have a low plasma adrenaline levels compared to age/sex matched controls and a degree of adrenomedullary dysplasia (Bornstein, Breidert et al. 1995). However, prior increase in cortisol has also shown to contribute to the defective counter-regulatory adrenaline responses. It has been suggested that the increase in cortisol by negative feedback, activates the type 2 glucocorticoid receptor, with a resulting downregulation of CRH and ACTH and decreased sympathoadrenal activation (Davis, Shavers et al. 1996).

Therefore, it can be speculated that the rise in cortisol in those with recurrent hypoglycaemia further contributes to the defective counter-regulation also likely to be seen in this group

In addition, there is some data suggesting that there is diminished cortisol feedback at the level of hippocampal/hypothalamic glucocorticoid receptors in streptozotocin (STZ) induced diabetes in rats. There is also often a lack of glucocorticoid inhibition with dexamethasone seen in this cohort (Roy, Collier et al. 1990).

There was however, no significant difference in CAR between the three groups. It is reassuring to know that although we calculated CAR based on one day of measurement, that studies have shown CAR to have a high intra-individual stability over 2 consecutive days (Hucklebridge, Hussain et al. 2005). ($r=0.63$ for the AUC_i). Accurate measurement of the CAR is highly dependent on subject compliance to the salivary protocol. Notably the first sample must be done immediately on waking. Any delay in this sampling may underestimate the rise in cortisol that is seen in CAR or even result in capturing the downward trend of cortisol after the initial burst of cortisol release. A study using polysomnography to determine waking have shown, the majority of individuals who do not display a CAR, are due to incorrect sampling. This showed that a delay of >15 minutes between wake time and first cortisol sample collection resulted in smaller CAR values (Okun, Krafty et al. 2010). However, even when sampling is done correctly, up to 10% of healthy cohorts have shown not to demonstrate a CAR. Two measures of dynamic increase were calculated- mean increase and AUC_i. The AUC_i makes the assumption that F45 is the peak sample. This is particularly a problem in a study involving mixed gender as in this study, as it is known that men's cortisol peaks at ≈ 30 mins post awakening, whereas women often peak later at ≈ 45 minutes (Pruessner, Wolf et al. 1997). It is known that both the increasing age and sex can affect the CAR. However in this dataset, neither of these factors was found to be interact with the CAR. Factors such as alcohol and smoking are known also to affect the CAR. To minimise this, subjects were advised to restrain alcohol intake for at least 24 hours prior to salivary sampling and current smokers were excluded.

Understanding the modulation of the HPA axis in T1D is currently limited, with most data derived from animal models. A study comparing Type 1 patients with and without retinopathy, suggested that the dysregulation (higher post-dexamethasone plasma levels of ACTH) was present in patients with moderate to severe retinopathy, and not in those with minimal to no retinopathy. It has previously been speculated that this may be as a result of microvascular damage in the limbic-HPA pathways which may have led to this dysregulation. However this difference was no longer significant, when duration of diabetes was incorporated (Roy, Collier et al. 1991).

However limited clinical studies show conflicting results. Overnight dexamethasone suppression tests in those with diabetes, are often non suppressed, particularly at 4pm rather than 8am, suggesting a prolonged hypercortisolaemic state in these individuals(Hudson, Hudson et al. 1984). However insulin has also been shown to promote several steps in the steroid synthetic pathway, with increased lipoprotein uptake and metabolism, increased levels of P450 side chain cleavage enzymes, as well as increased enzymatic activity of 3β hydroxysteroid dehydrogenase, 21 and 11β hydroxylase(Ghizzoni, Vanelli et al. 1993) and may also contribute to this hypercortisolaemic state. However, glucocorticosteroids are also known for their anti-insulin action on peripheral tissues. We find in our study that increased exposure to hypoglycaemia can further contribute to this hypercortisolaemic state. This state could have the potential to contribute or worsen the vascular complications of diabetes.

Limitations

One of the main limitations of this study is the cross-sectional nature of data collection, and therefore no conclusion can be made on the long term impact of a potential dysregulated HPA axis. Studies have suggested asking the subjects to collect saliva over multiple days. However, they have often asked subjects to collect less samples if collecting on multiple days

(e.g. wakening, 30min and before bed) to ensure sampling compliance. Our approach was a more detailed look at the slope, and hence we chose the multiple samples-single day approach. Reassuringly, a previous study(Edwards, Clow et al. 2001) utilising sampling over 2 consecutive days showed both the AUC of the wakening period (0-45mins) and the slope of the decline showed reasonable stability over the 2 days of sampling, suggesting that a single day of sampling as in our study, may be adequate and representative.

Even with a day of salivary cortisol sampling, it is nevertheless a cross sectional analysis and reveals more acute changes. Recently analysis of scalp hair cortisol (with a growth rate of approximately 1cm/month with the most proximal part growing in the last month) has been shown to be a promising technique for the retrospective assessment of average cortisol exposure over a few months(Lee, Kim et al. 2015).

Other exploratory studies on this dataset

Correlation between HbA1c and GV parameters

Over the last 40 years, HbA1c has been understood as reflecting mean blood glucose over the previous 2-3 months. However indicators of GV could provide information about other aspects, revealing patterns of high and low excursions and indicators of lability. Studies have looked at comparing HbA1c with self-monitoring of blood glucose (SMBG) over a 3 month period. As expected a close correlation was shown between HbA1c and mean blood glucose from SMBG ($r=0.62$), and within subject SD was also shown to be correlated with HbA1c ($r=0.375$)(Derr, Garrett et al. 2003). A recent study also examined the relationship between HbA1c and various GV parameters in a study which utilised CGM for at least 48 hours, which included 35 patients with T1D. Although T2D were included, HbA1c correlated with mean glucose ($r=0.74$) and AUC PP (postprandial index) only in those with T1D. HbA1c

was shown not to correlate with LBGI, further demonstrating the inability of HbA1c of identifying those with recurrent hypoglycaemia or indeed predicting those at risk of future hypoglycaemia (Sartore, Chilelli et al. 2012). In our GV cohort, we used linear regression to determine if there was a relationship between HbA1c and CGM-derived GV indices.

Table 23 Correlation coefficients between HbA1c and Glycaemic variability indices.

| Index of GV | R2 | P value |
|--------------------|-----------|----------------|
| Mean | 0.390 | <0.0001 |
| SD | 0.163 | <0.001 |
| CONGA | 0.411 | <0.001 |
| LI | 0.012 | 0.275 |
| J-index | 0.396 | <0.001 |
| LBGI | 0.00032 | 0.860 |
| HBGI | 0.371 | <0.001 |
| GRADE | 0.382 | <0.001 |
| MODD | 0.106 | 0.0009 |
| MAGE | 0.005 | 0.467 |
| ADRR | 0.202 | <0.001 |
| M value | 0.345 | <0.001 |
| MAG | 0.017 | 0.191 |

Univariate analysis showed that there was a close association between HbA1c and indices of average BG (Mean, SD, CONGA, J-index, GRADE, and MODD). It was a poor indicator of the burden of hypoglycaemia as assessed by LBGI. HbA1c was also a good reflection of chronic hyperglycaemia (HBGI), there was also a good correlation to ADRR (a measure of total variability accounting for highs and lows), but it is likely that this is because of the strong correlation between HBGI and ADRR.

Correlation between GV and insulin doses

Table 24; Correlation coefficients between total insulin doses and measures of Glycaemic variability.

| Index of GV | R ² | P value |
|-------------|----------------|--------------|
| Mean | 0.05 | 0.028 |
| SD | 0.006 | 0.454 |
| CONGA | 0.052 | 0.025 |
| LI | 0.002 | 0.685 |
| J-index | 0.033 | 0.076 |
| LBGI | 0.0003 | 0.85 |
| HBGI | 0.028 | 0.101 |
| GRADE | 0.062 | 0.01 |
| MODD | 0.008 | 0.384 |
| MAGE | 0.0001 | 0.912 |
| ADRR | 0.189 | 0.190 |
| M value | 0.018 | 0.194 |
| MAG | 0.003 | 0.588 |

Univariate analysis showed that insulin doses are associated with GV indexes reflecting mean glucose (mean, CONGA, GRADE), suggesting that a greater dose of insulin is associated with a poorer glycaemic control. This is suggestive that this greater dose of insulin is taken inappropriately by those with poor glycaemic control, for example, possibly through taking large corrective doses to overcome hyperglycaemia.

CHAPTER 7:

STUDY 4; CRH testing in Type 1 Diabetes

Background

Baseline tests (such as the cortisol curve) are often simpler to perform than dynamic tests, however they have appeared to lack reliability in pharmacological terms. Dynamic testing with either stimulation or inhibition have been shown to be far more reliable, with greater between person variability, and a higher reliability (Coste, Strauch et al. 1994). We therefore in this follow on study, investigated the effect of Corticotrophin-releasing hormone (CRH) on the HPA axis in those with T1D to determine if there is a dysregulation of the axis due to recurrent hypoglycaemia. It is well established that insulin induced hypoglycaemia (IIH) is a powerful stimulant of CRH, however, what is yet to be reported, is whether, RH and therefore recurrent stimulation of CRH causes blunting or dysregulation of the whole axis.

CRH, a 41 amino acid peptide is a principal regulator of the HPA axis (Axelrod and Reisine 1984) and is also involved with regulating the autonomic, immunological and behavioural response to stress (Owens and Nemeroff 1991). CRH neurons are localised through the cortex, limbic system and brainstem, which are all associated with autonomic functioning. High concentrations are found in the paraventricular nucleus of the hypothalamus and the hypophyseal portal system. CRH interacts with its receptor on the pituitary corticotrophs, which leads to increased formation of cAMP, leading to a cascade of events which ultimately results in secretion of POMC derived peptides into the peripheral circulation. (Owens and Nemeroff 1991). CRH interacts with two G protein coupled receptors, CRH1 and CRH2. CRH1 appears to be the major receptor mediating the activation of the HPA axis in rodents. Mice deficient in CRH1 show adrenal atrophy and a reduced corticosterone stress responsiveness, whereas CRH2 null mice present with normal corticosterone responses but an

altered recovery phase(Yoshida-Hiroi, Bradbury et al. 2002). CRH1 appears also to be crucial in normal chromaffin cell development, and CRH1 null mice have also been shown to have an impairment in adrenal catecholamine production (Yoshida-Hiroi, Bradbury et al. 2002). Early studies have showed that synthetic intravenous CRH produced vasodilation and hypotension and central administration showed an increase in mean arterial pressure (MAP) and heart rate which is well known to be separate from activation of the HPA. Increases in MAP usually cause reduction in heart rate, however under stressful conditions, the baroreflex function can be altered enabling simultaneous rise in both parameters. Central administration of CRH does not affect baroreflex sensitivity, but has been shown to increase sympathetic and decrease central parasympathetic outflow. (Overton, Davis-Gorman et al. 1990). CRH selectively stimulates pituitary corticotropin (ACTH) secretion in man. Other neuro-regulators such as oxytocin and adrenaline have also been found to have ACTH releasing properties as demonstrated during immune-neutralisation of CRF(Carnes, Lent et al. 1990). Vasopressin was also found to stimulate ACTH secretion but also potentiates the action of CRH on the anterior pituitary. Insulin induced hypoglycaemia has not only been shown to activate CRH but has been shown to increase plasma vasopressin concentrations. It has been shown there is a strong correlation between the depth of hypoglycaemia and the rise in plasma vasopressin. ($r=0.57$ $p<0.001$). A study in humans showed that the release of vasopressin in this context was independent of blood pressure and volume changes, and as yet, the mechanism is unknown(Baylis, Zerbe et al. 1981).

Although the effect of acute CRH stimulation is well established, what is less well known is whether recurrent stimulation of CRH can produce the same response. A rodent study showed that continuous infusion of CRH caused a 40% decrease in the anterior pituitary CRH receptor concentration. However when there was a concomitant infusion of vasopressin (at a dose resembling the concentration of vasopressin in the pituitary portal circulation), the

effects of CRH on the anterior pituitary was exaggerated. (I.e. greater reduction of CRH receptor concentration.). Vasopressin is both a neuroendocrine hormone (located in the magnocellular neurons of the hypothalamus) but also a neurotransmitter (located in the parvocellular suprachiasmatic nucleus), and the study showed that it is the activation of the parvocellular component rather than the magnocellular component, which was crucial in potentiating the down-regulatory effects of CRH on the anterior pituitary CRH receptors(Hauger and Aguilera 1993).

The impact of chronic or excessive exposure to CRH has been debated in the literature for a number of years. Both in-vivo and in-vitro studies have shown that prolonged CRH receptor occupancy increases the POMC peptide synthesis, with trophic actions of the pituitary (Westlund, Aguilera et al. 1985, McNicol, Kubba et al. 1988). However, in contrast other studies shown that continuous or excessive exposure to CRH cause a desensitisation. These studies show reduced ACTH secretion in continuous exposure, however CRH still stimulated ACTH above baseline (Rivier and Vale 1983, Evans, Brett et al. 1985).

CRH stimulation tests

CRH has been established as providing a prominent role in orchestrating the response to stress. Intravenous CRH has been shown to have an apparent volume of distribution equal to that of plasma volume and a final eliminating half-life ranging from 45 to 180 minutes.(Schulte, Chrousos et al. 1985). The threshold dose has been shown to be 0.03mcg/kg, with the maximal effective doses between 3-10mcg/kg. Doses over 0.3mcg/kg of CRH show a peak ACTH after 10-15 minutes, with plasma cortisol levels reaching a peak at 30-60 minutes. The responses to ovine CRH (1mcg/kg) have also been found to vary depending on the time of administration. Later (8pm vs 9am) administration of CRH resulted in ACTH responses being slightly higher, however the cortisol response was significantly greater in the evening. ($P<0.005$).

Haemodynamic parameters were unaltered in this study, however 10% of patients reported an upper body and facial flush. (Schulte, Chrousos et al. 1985). This flush often begins almost immediately and lasts between 3 to 5 minutes. A large study showed 6% also reported an urge to take a deep breath. Doses often over 3mcg/kg were associated with more severe symptoms such as tachycardia, hypotension, dyspnoea and a chest tightness(Nink, Krause et al. 1991).

Studies so far in those with Type 1 Diabetes

Roy et al, (Roy, Roy et al. 1993) examined the CRH response in 22 subjects who had T1D with no history of depression, and showed total plasma integrated ($p<0.004$) and peak cortisol levels($p<0.002$) were significantly higher in those with T1D compared to age/sex/BMI matched controls, however plasma ACTH was not significantly different between the groups. Roy et al, also found there was a trend ($p<0.1$), of patients with any complications (retinopathy, neuropathy or both) having a higher peak plasma cortisol to CRH

than subjects without complications. These differences were not attributed to changes in cortisol binding globulin (CBG). Specifically looking at neuropathy in those with Type 1 Diabetes, Cairo et al (Cairo, Volpi et al. 1995), examined 3 groups (controls, Type 1 with neuropathy and Type 1 without neuropathy) and found that when CRH ($0.03\mu\text{g/kg}$) (a dose which would give an accurate evaluation of the HPA and a plasma level of CRH equivalent to that in the hypophyseal portal circulation), the ACTH response was significantly higher ($F=6.73$, $p<0.02$) in non-diabetic control subjects, and within the groups with diabetes, ACTH response was greater in those without neuropathy, compared to those with neuropathy. ($F=5.43$, $p<0.05$). The cortisol response was found to be significantly lower in non-diabetic controls as opposed to the 2 diabetes groups and within the diabetes groups, higher levels of cortisol were found within the group with neuropathy. This would suggest that neuropathy exacerbates an already abnormal HPA axis.

Hypothesis for dysregulation of the HPA axis

HPA hyperactivity

This has been recognised as an elevated circulating level of cortisol and/or ACTH (corticotrophin) and a resistance to dexamethasone suppression. (Cameron, Kronfol et al. 1984, Roy, Collier et al. 1990) Several hypothesis have been reported in the literature as to the reason for this, and detailed below.

Increased hypothalamic drive

Rodent models have shown a reduction in ACTH response to CRH, and this was shown to be as a result of a decreased number of CRH receptors in the anterior pituitary, which then led to greater CRH hypersecretion and therefore an increased hypothalamic drive. (Scribner, Walker et al. 1991)

*Downregulation of CRH receptors in the anterior pituitary-
Decreased sensitivity of pituitary*

Studies using a higher dose of CRH 1µg/kg are thought to be useful to ascertain pituitary ACTH storage, however lower doses of CRH are needed to assess pituitary sensitivity.(Coiro, Volpi et al. 1995) Studies using the low dose of CRH, have shown there is a reduced(Coiro, Volpi et al. 1995) (Schulte, Chrousos et al. 1985)or unaltered(Roy, Roy et al. 1993) ACTH release with CRH stimulation suggesting pituitary insensitivity. This tolerance appears to be at the level of CRH receptor and its coupling to adenylate cyclase. Chronic CRH infusion for 48 hours has shown a 46% decrease in CRH receptor binding (Wynn, Harwood et al. 1988).

Decreased glucocorticoid negative feedback sensitivity

It has been postulated that in those with diabetes, the HPA axis has decreased sensitivity to the negative feedback of glucocorticoids. This has been seen in the poor suppression of glucocorticoids with dexamethasone (25mcg/kg) suppression tests seen in diabetic animals (Chan, Inouye et al. 2002) and in patients with diabetes. (Hudson, Hudson et al. 1984)

Lack of blunting of ACTH.

Roy et al(Roy, Roy et al. 1993), showed that ACTH levels in the context of high cortisol levels were either normal or just slightly increased, and therefore there is a lack of blunting of ACTH suggesting dysregulation of the HPA axis. This is a similar pattern to those who are severely ill intensive care patients and those who are under chronic physical stress, also show normal or exaggerated response of the corticotroph to CRH, nevertheless with high levels of endogenous glucocorticoids(Reincke, Allolio et al. 1993).

Direct stimulatory effect of CRH on the adrenals

Roy et al also proposed that CRH and/or ACTH had an exaggerated effect on the adrenals, with CRH having a potential direct action on the adrenals. It has been proposed this may be due to adrenal hypertrophy, which is seen after a few days of excessive stimulation with ACTH(Symington, Duguid et al. 1956). This is also seen in diabetic mice, showing an increase in the size of the adrenal glands(Carson, Hanker et al. 1982). However CRH has also been shown in rodent models to have a direct trophic response to the chromaffin cells in the absence of pituitary ACTH, suggesting a close link between the HPA axis and the neuroendocrine system.(Hoheisel, Schauer et al. 1998)

Hyperinsulinaemia

Hyperinsulinaemia is commonly seen in those with T1D, and it has been suggested that these high insulin levels may stimulate the adrenal medulla via binding of insulin to its own adrenal receptors. Euglycaemic clamp studies using high dose (15 mU/min/kg) and low dose (1.5 mU/min/kg) insulin infusions showed that CRH stimulation during euglycaemia resulted in an ACTH response only in the high dose insulin subgroup ($p<0.01$) and serum cortisol levels were also found to be significantly greater only in the high dose insulin group ($p<0.02$)(Fruehwald-Schultes, Kern et al. 1999) Roy et al also found that greater insulin doses were associated with greater perturbation of the HPA axis. (Roy, Roy et al. 1993)

Poor glycaemia control

Poor diabetic control is associated with hyper activation of the hypothalamo-pituitary-adrenal (HPA) axis. (Cameron, Kronfol et al. 1984) Wurzbürger et al found that better glycaemia control was related to significantly lower 24 hour cortisol secretion profile in those with T1D(Wurzbürger, Prelevic et al. 1990).

Hypoglycaemia and the HPA axis

Early reports have confirmed that insulin induced hypoglycaemia (IIH) is a powerful stimulant of ACTH production with CRF (permissive role)(Plotsky, Bruhn et al. 1985) and vasopressin (modulatory role) accompanying this response(Plotsky, Bruhn et al. 1985). In a pilot study of non-diabetic healthy men, IIH produced peaks in CRH, ACTH and AVP at 45 minutes after insulin injection, and maximal cortisol responses were seen at 90 minutes(Ellis, Schmidli et al. 1990). Studies have shown that IIH can also stimulate production of adrenal E, this was shown to stimulate production of hypothalamic E, found also to be a stimulator of ACTH via β adrenergic receptors found on the pituitary, thereby suggesting both central and peripheral pathways involved in activation of HPA activity(Tilders, Berkenbosch et al. 1982). It may be that the magnitude of hypoglycaemia impacts the pathway activated with more profound hypoglycaemia activating peripheral mechanisms and vice versa (Aizawa, Yasuda et al. 1981). There is very little literature about the specific impact of recurrent hypoglycaemia on the HPA axis. We know that a 2 hour episode of acute hypoglycaemia to 3.0mmol/L is usually required to diminish the cortisol response to further hypoglycaemia. (Davis and Shamoon 1991, Veneman, Mitrakou et al. 1993, Fruehwald-Schultes, Kern et al. 1999). However it appears that the diminished response of cortisol to further hypoglycaemia are stimulus specific as illustrated by a study (Welt, Kinsley et al. 1998), which assessed the HPA axis in those with recurrent hypoglycaemia by using a non-hypoglycaemic stimulus (ACTH) and found that there is an exaggerated cortisol response to ACTH. This was proposed to be as a result of some adrenal gland priming due to endogenous ACTH release during prior hypoglycaemia and the exogenous ACTH. This was confirmed in a study that gave repeated doses of ACTH, which resulted in a significantly increased cortisol response during the second exposure to ACTH. (Kolanowski, Pizarro et al. 1975)

Methods

The last 30 subjects in the (GV and cortisol study-Study 3) were recruited to perform an exploratory study to examine whether RH (through recurrent stimulation of CRH) in subjects with T1D affected the response to further exogenous CRH. The pituitary and adrenal responses to CRH were examined by measuring ACTH and cortisol responses after intravenous administration of CRH.

10 subjects each with high LBGI, low LBGI and normal individuals with no endocrine disorders were studied. All subjects were informed of the purpose of the study, and gave their written informed consent. Following discussion, strategies were also implemented so that those with T1D, would not have a hypoglycaemic episode from the afternoon (12pm till after the conclusion of the CRH stimulation test). This was done so that there would be no other stimulus for cortisol release in the afternoon/evening on the day of the test.

Subjects were asked to attend the clinical research centre (CRC), Ninewells, at a time between 4 and 6pm. On arrival, an intravenous cannula was inserted into the ante-cubital vein of the non-dominant arm. Baseline bloods were only done at least 30-60mins after the insertion of the cannula. (to overcome effects of cannulation (a potential stressor) on cortisol). Bloods were taken for ACTH and cortisol. Plasma ACTH (ELISA, ALPCO) and cortisol (radioimmunoassay, DIASORIN) concentrations were measured in all samples. Cortisol samples had an inter-assay CV -7.4% and intra-assay CV -7.2% with a sensitivity of 5.88nmol/L. ACTH had an inter-assay CV -6.2% and intra-assay CV-2.6% with a sensitivity of 0.22pg/ml. Blood glucose and blood pressure were also checked every 10minutes for the cohort with diabetes. Blood glucose was only checked at the beginning and end of the study in the non-diabetic cohort. Baseline bloods were taken 15 (TP1) and 5 (TP2) minutes before CRH administration. Synthetic ovine CRH was administered as a slow intravenous bolus at a

dose of 1mcg/kg. Blood samples were then taken at 15 minute intervals for the first hour (TP3-6), and then at 90 (TP7) and 120 (TP8) minutes. Subjects were given a meal at the end of the study and then discharged.

Based on a similar study looking at patients with Type 1 Diabetes and neuropathy (Coiro, Volpi et al. 1995) and assessing the cortisol and ACTH responses to intravenous CRH in patients with T1D, we found we would need to recruit 10 subjects in each group accounting for a 20% drop out rate, to give us 90% power, to detect α of 0.05.

Results

Outliers

Any results (ACTH or cortisol) which were out with of 2 SD were removed from the dataset. If a subject had 3 or more outliers, then that subject was also removed from the final analysis.

One subject was removed from the high group because of 5 ACTH outliers. Examining the demographics of this subject, age, duration of diabetes and glycaemic control, there were no differences in these parameters compared to the rest of the group. However this was the only subject in this group, who had laser photocoagulation for proliferative diabetic retinopathy. Therefore, this result requires further exploration. Could it be hyper-functioning of the HPA axis contributed to the development of this chronic complication or is presence of diabetic retinopathy a required condition for increased HPA activity?

Two subjects were removed from the low group, the first subject was removed because therapy had changed prior to fitting the CGM, such that CGM did not reflect prior exposure to hypoglycaemia, so LBGI calculation could not be used. 2nd subject was removed because of 4 ACTH readings which were out with 2 SD. No explanation could be found in this subject, for these abnormal results.

1 subject was removed from the control group because there were 5 ACTH readings out with 2SDs.

Table 25 The final numbers of subjects out of 10 who were studied as part of the CRH stimulation study.

Reasons for exclusion are detailed above.

| | High | Low | Control |
|-----------------------|------|-----|---------|
| Final number analysed | 9 | 8 | 9 |

None of the T1D had any major microvascular complications (>mild BDR, >microalbuminuria, >subclinical neuropathy) or macrovascular complications.

Table 26; Demographics of each group examined during the CRH stimulation tests.

| | High | Low | Control | |
|----------------------|------------|------------|------------|-----------------------------|
| Age | 39.6 (4.6) | 46.5 (4.6) | 43.8 (3.6) | F (2, 23) = 0.6647 P = 0.52 |
| Duration of diabetes | 27.2 (3.5) | 16.2 (2.9) | n/a | P=0.03 |
| Glycaemic control | 8.4 (0.3) | 8.8 (0.4) | n/a | P=0.37 |
| LBGI (mean(SEM)) | 8.6(0.6) | 2.8(0.4) | n/a | P<0.0001 |

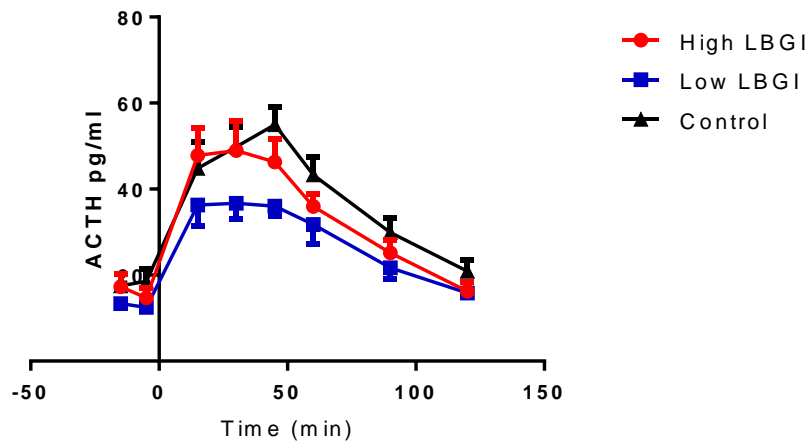
ACTH parameters

Does hypo frequency affect ACTH response in T1D?

After CRH (1mcg/kg), the rise in ACTH is shown in the graph below

Figure 26; This shows the ACTH response from baseline to after stimulation with CRH.

Intravenous CRH is given at time 0.



The mean (SEM) baseline ACTH (taken from TP2), was (H v L v C) (14.7 (2.4) vs 12.5 (1.3) vs 18.6 (2.9)). (Kruskal Wallis statistic 3.77, $p=0.15$). This showed that baseline ACTH was not statistically different in the 3 groups.

The rise in ACTH following CRH administration was calculated by subtracting the baseline ACTH from peak ACTH. Firstly, there was no effect of grouping on the change in ACTH. $F(2,23) = 2.17$ $p=0.137$. Two way ANOVA showed there was no statistical significant interaction between the effects of group and duration of diabetes on change in ACTH $F(1,12) = 0.028$ $p=0.869$. There was also no effect of group and glycaemia on change in ACTH $F(2,10) = 0.469$ $p=0.639$.

Table 27 This shows basal and maximal response (peak) and the maximal rise (Δ) in ACTH responses between the 3 groups

| ACTH | High | Low | Control |
|--------------------|------------|------------|------------|
| Basal (mean (SEM)) | 14.7 (2.3) | 12.5 (1.1) | 18.5 (2.8) |
| Peak | 53.1 (5.9) | 41.8 (3.0) | 59.0 (4.4) |
| Δ | 38.4 (3.6) | 29.3 (1.9) | 40.5 (1.7) |

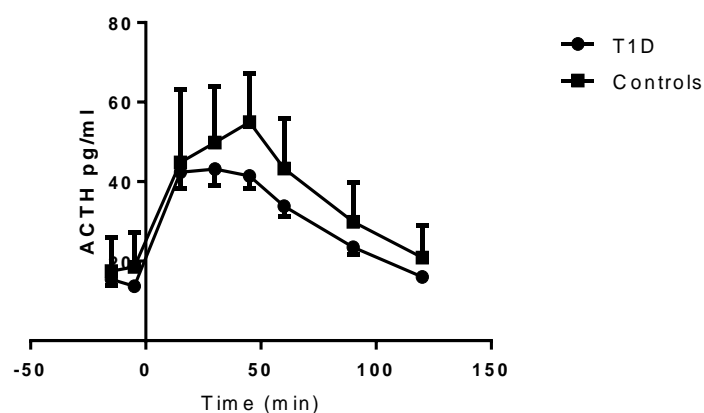
A two-way ANOVA was conducted to examine the effect of group and duration of diabetes on the peak ACTH. There was no statistically significant interaction between the effects of group and duration of diabetes on peak ACTH. $F(1, 12) = 0.019$ $p=0.893$. A further two way ANOVA looked at the effect of group and glycaemia on the peak ACTH. Again there was no statistically significant interaction between the effects of group and glycaemia on the peak ACTH $F(2,10) = 0.256$ $p=0.779$.

The mean (SEM) area under the curve (AUC) of the ACTH curve was (H v L v C) (4441 (476) vs 3583(326) vs 4907 (369)). Although the AUC of the high group was higher than the low group, this was not statistically significant. ($p=0.09$). One way ANOVA, showed no significant difference between the 3 groups $F(2,23)=1.37$ $p=0.27$. Two way ANOVA, showed no statistically significant interaction between group and glycaemia on the AUC (ACTH). $F(2,10) = 0.693$ $p=0.523$. There was also no significant interaction between group and duration of diabetes on the AUC (ACTH) $F(1,12)=0.472$ $p=0.505$.

Does having T1D affect ACTH response to CRH stimulation?

Figure 27; ACTH response following CRH stimulation.

CRH administered at time 0 in the combined group with Type 1 Diabetes (low LBGI + high LBGI) T1D ($n=17$) and in the control cohort ($n=9$)



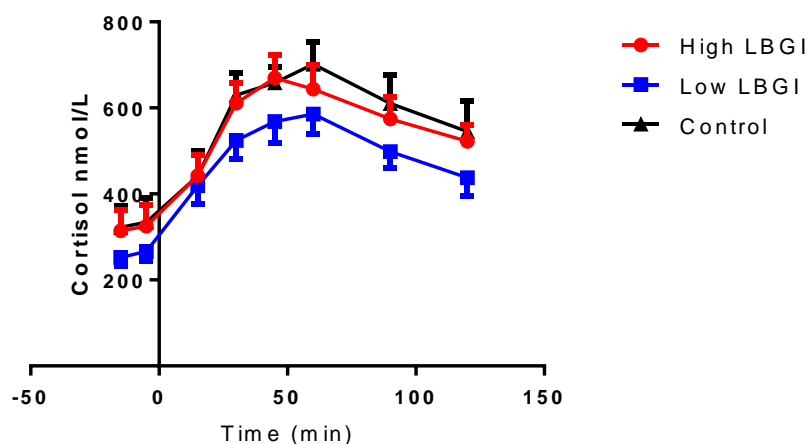
There was a trend towards reduction in peak ACTH response in those with T1D compared to the control subjects however it did not reach significance. (D vs C) (47.7 (3.8) vs 59.0 (4.7). (unpaired t test $p=0.08$) There was also no significant change in AUC (T1D-68809, Controls-75951 $p=0.63$)

Cortisol parameters

Peak cortisol was highest in the control group compared to the diabetic groups, however this did not reach statistical significance $F(2,23)=1.883$ ($p=0.17$). Two way ANOVA showed that there was no statistically significant interaction of group or diabetes duration on the peak cortisol $F(1,12)=0.955$ $p=0.348$. There was also no significant interaction between group and glycaemia on the peak cortisol $F(2,10)=0.654$ $p=0.541$.

Figure 28; This shows the cortisol response from baseline to after stimulation with CRH.

Intravenous CRH is given at time 0.



The rise in cortisol from baseline was highest in the control group however this was not statistically significant $F=0.5253$ $p=0.60$. Two way ANOVA showed that there was no

statistically significant interaction of group or glycaemia on the rise in cortisol $F(2,10)=0.125$ $p=0.884$. There was also no statistically significant interaction of either group or diabetes duration on the rise in cortisol $F(1,12)=1.416$ $p=0.257$.

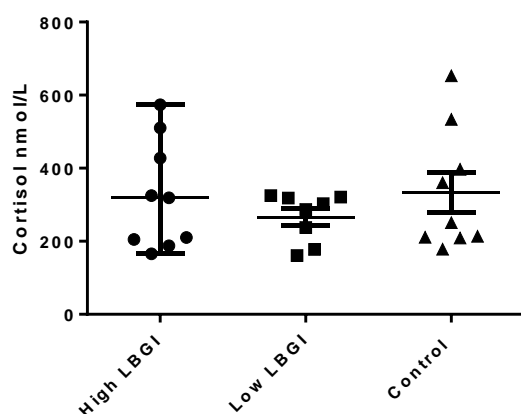
Table 28; This shows basal and maximal response (peak) and the maximal rise (Δ) in cortisol responses between the 3 groups

| Cortisol (nmol/L) | High | Low | Control |
|-------------------|--------------|--------------|--------------|
| Basal | 325.0 (47.3) | 266.3 (20.9) | 334.0 (52.8) |
| Peak | 694.1 (51.2) | 590.6 (48.6) | 736.6 (50.1) |
| Δ | 369.2 (4.0) | 324.3 (60.0) | 402.6 (59.7) |

There was no significant difference between the AUC (cortisol) between the 3 groups (H vs L vs C) (73386 (5932) vs 63661 (4072) vs 75951 (6476). $F(2,23)=1.243$ $p=0.307$. Two way ANOVA showed no statistically significant interaction between group and glycaemia on the AUC (cortisol) $F(2,10)=1.039$ $p=0.389$. There was also no statistically significant interaction between group and duration of diabetes on the AUC (cortisol) $F(1,12)=1.3$ $p=0.277$.

Baseline cortisol (TP2) did not differ between the 3 groups (325 (49.8) vs 266.3 (23.4) vs 334 (55.6). $F=0.6$ ($p=0.56$). However two subjects in the control group had basal cortisol levels outside of 2 SDs, were thought to be stressed, based on their basal cortisol levels.

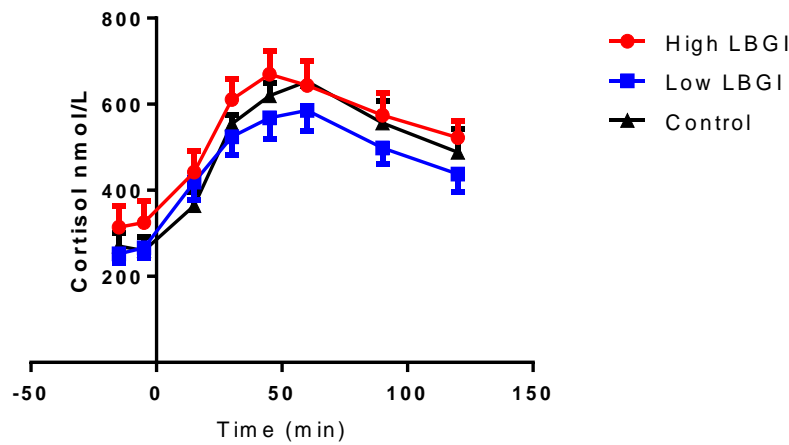
Figure 29; Box and whiskers plot showing the scatter of the baseline cortisol levels in the 3 groups.



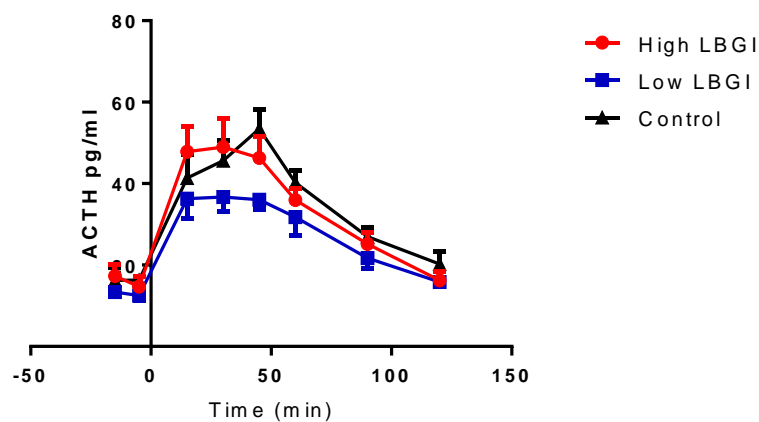
The profile of ACTH and Cortisol release are shown with these 2 subjects removed.

Figure 30; Cortisol and ACTH responses following CRH stimulation.

a. Cortisol and b. ACTH responses following CRH stimulation at time 0, with the 2 outliers in the control group removed.



ACTH to draw graph- 3 groups

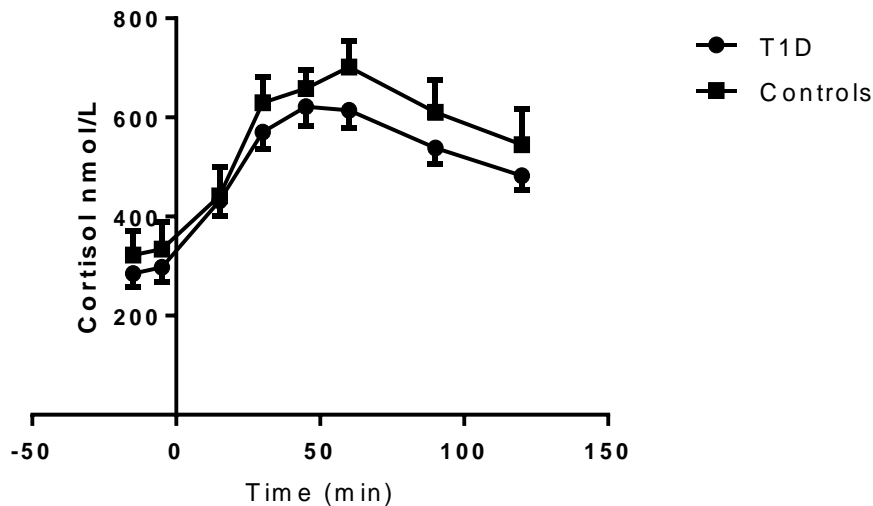


All the analysis, were carried out with these 2 subjects removed to determine if removing these outliers had an impact on the results. There was still no difference in peak cortisol $F(2,21)=1.176$ $p=0.3281$, AUC of cortisol $F(2,21)=1.066$ $p=0.3623$ or rise in cortisol $F(2,21)=1.652$ $p=0.2156$.

Does T1D affect cortisol response to CRH?

Figure 31; Cortisol responses following CRH stimulation in those with Type 1 Diabetes (T1D) compared to non-diabetic control subjects.

This illustrates the cortisol response following CRH administration at time 0.



There was a non-significant decrease in peak cortisol responses to CRH stimulation in those with T1D. (D v C) (645.4 (39.3) vs 736.6 (52.8). $p=0.1818$).

Discussion

These results show a trend (however non sig at $p=0.08$) towards a lower ACTH response in T1D compared to controls (it is thought that plasma ACTH is a more sensitive marker of the central regulation of the HPA axis, rather than plasma cortisol, which is partly dependant on the adrenal sensitivity to steroids), with no difference in the cortisol response between the 3 groups. Subdividing the T1D group further by exposure to hypoglycaemia did not reveal any further trends. These results do not suggest major dysregulation of the HPA axis in subjects with T1D.

If the trend towards a lower ACTH, were likely to increase to significance, with increased numbers of subjects, then this is consistent with other studies in T1D showing a lower ACTH

response to CRH stimulation. Lower ACTH responses to CRH may indicate a pituitary insensitivity.

We may have missed a true result of pituitary insensitivity in this circumstance, by giving too high a dose of CRH. A similar (normal ACTH responses to CRH) was seen in another study using a similar dose (1mcg/kg) (Roy, Roy et al. 1993), but a study using a threshold dose of CRH (0.03mcg/kg)(Coiro, Volpi et al. 1995) did show poor ACTH responses in those with T1D. It has been shown that in order to truly study pituitary sensitivity, a minimal effective dose (0.03mcg/kg) of CRH should be used. This has been shown to be the threshold dose which produced a plasma CRH in the reported range in the hypophyseal portal circulation. This threshold dose was shown to stimulate with reproducibility POMC peptide secretion from the anterior pituitary(Watson, Lopez et al. 1988). The maximal dose (such as 100mcg/or 1mcg/kg such as in this study), may just cause release of pre-stored ACTH from the pituitary corticotrophs rather than truly test pituitary sensitivity. However dose-response studies have shown that 1mcg/kg is the lowest dose at which maximal cortisol stimulation occurs. We may have been able to differentiate further by conducting two studies using 0.03mcg/kg to look at pituitary sensitivity, and possibly a higher dose i.e. 1mcg/kg to look at adrenal responsiveness.

A number of explanations could be given for a possible reduction in ACTH responses in those with T1D. STUDY 3 investigated diurnal cortisol rhythms in those with T1D. I showed that there was a blunted cortisol decline as well as a greater mean AUC of cortisol from 3-12 hours post wakening, through the day in patients with T1D, and therefore greater exposure to cortisol, may result in enhanced feedback control over corticotropin (ACTH) peptide release.

An alternative explanation, patients with T1D may also be exposed to chronic overstimulation of CRH release due to a number of factors such as hypoglycaemia, poor glycaemic control and even exogenous insulin's action on the hypothalamus. Rodent studies have shown chronic overstimulation of CRH in diabetic animals leads to a significant downregulation of CRH receptors in the anterior pituitary, resulting in a reduced ACTH response (Scribner, Walker et al. 1991).

The latter explanation is more likely in this scenario, as the basal levels of cortisol for the subjects with diabetes did not differ between the three groups. $F(2,23)=0.5983$ $p=0.5581$. Removing the 2 possible control subjects who were stressed, showed there was still no difference in the basal cortisol in the T1D group compared to the controls (297.4 (28.7) vs 259.9 (31.9), $p=0.4571$). A previous similar study (Roy, Roy et al. 1993) showed that there was no difference between T1D and controls in the evening, when basal cortisol levels are low, but during the morning, the cortisol was significantly higher in diabetic group. Rather it maybe the raised cortisol during the day (as suggested by STUDY 3), that has more impact on the suppression of corticotrope response to CRH in those with T1D. We chose to study our subjects in the evening, because a series of experiments in human subjects have shown that amplitude of cortisol pulses are greatest in the early hours after wakening, with the amplitude decreasing over the course of the day, with the lowest in the evening. It was suggested (by Lightman, SL) that in order to assess the response to exogenous oCRH with minimal variability, the experiment should be carried out in the evening after 5pm. The response to CRH could show much variability in the morning/afternoon, when the amplitude of endogenous cortisol release is greatest as the responses may be affected by whether the cortisol was in the upward or downward phase of the secretory response. Therefore in order to minimise variability in ACTH and cortisol responses following intravenous injection of CRH, all studies were carried out in the evening hours (after 5pm), when theoretically the

levels of endogenous cortisol and amplitude would be at its lowest. This would be a time period where the amplitude of cortisol secretion would be low, and therefore, there would be minimal variability in the cortisol and ACTH responses. However by doing the study in the evening, we may be able to avoid variability, but may not pick up the maximal cortisol response compared to a morning study.

In this study, there was no difference in the cortisol response to CRH between the three groups. This is in contrast to a study in adolescents (Ghizzoni, Vanelli et al. 1993) whereby a 1mg/kg CRH stimulation study led to a significant increase in plasma cortisol. The authors suggest that this may be due to the metabolic status of diabetes, with consequently a stress related change in the overall physiology leading to a preferential production of steroids and cortisol. This study also did not show any change in ACTH, suggesting an absence of a centrally mediated mechanism. It was suggested that the increased cortisol production was rather a peripherally driven mechanism. Mechanisms proposed include a direct action of insulin on specific adrenal receptors (Penhoat, Chatelain et al. 1988) and a paracrine activation of an intraadrenal CRH/corticotropin mechanism. It appears that this intra-adrenal system (in rodent studies) is particularly active during a central alteration of the CRH/ACTH branch (during hypophysectomy), possibly through a negative feedback mechanism (Mazzocchi, Malendowicz et al. 1994). This system does not appear to provide adequate replacement, as demonstrated by the essential need of hydrocortisone replacement in those patients with hypopituitarism. The adrenal zona medullaris of several species has been shown to co-release CRH with catecholamines (Suda, Tomori et al. 1986). Rodent studies have shown that high dose CRH can stimulate secretion of corticosterone which is independent of ACTH release by the pituitary (Andreis, Neri et al. 1991). It may therefore be that there is a chronic activation of CRH in patients with diabetes either as a result of the metabolic state or through intermittent activation of CRH during times of particular stress

(extreme glucose variability) leading to high peripheral cortisol levels. Presence of neuropathy in the threshold CRH study (0.03mcg/kg) was shown to further suppress the ACTH responses to stimulation.

Abnormal responses to CRH have been shown in a number of pathological states. For example in Cushing's syndrome, those with a pituitary Cushing's had an exaggerated response of ACTH and cortisol to CRH, whereas those with adrenal or ectopic disease had a blunted response of ACTH and cortisol release following CRH administration. (Chrousos, Schulte et al. 1984). This may be due to an enhanced and appropriate negative feedback on the corticotropes. Several psychiatric disorders such as depression(Gold, Loriaux et al. 1986) and anorexia nervosa(Gold, Gwirtsman et al. 1986) and those with panic disorder(Roy-Byrne, Uhde et al. 1986) have shown a basal hypercortisolaemia associated with a blunting of ACTH response to CRH stimulation, which suggests an appropriate negative feedback on the corticotropes, which is in contrast to the response in patients with pituitary Cushing's.

A major limitation of this study is the low numbers of subjects. However this was a pilot study to determine if there was initially a signal before conducting a larger scale study.

CHAPTER 7; Conclusions and opportunities for further research

90 years have passed since the discovery of insulin and despite all the advances in insulin therapy, delivery devices and education, there still remains a “dark side to this potent preparation” which is hypoglycaemia. The frequency of hypoglycaemia has not really changed in the past few decades, and continues to contribute to the significant morbidity and mortality associated with T1D.

Chapter 1 looked at the problem and epidemiology of “hypoglycaemia” in those with Type 1 Diabetes (T1D) commenting on the particular risk factors related to recurrent hypoglycaemia. The likely mechanisms responsible for the development of defective counter-regulation during hypoglycaemia in those with T1D were reviewed. On average, a person with T1D experiences 2 or 3 mild symptomatic hypoglycaemia every week, and the consequences of recurrent hypoglycaemia on various physiological states were reviewed.

In chapter 2, both non-drug and drug strategies when used as adjunct therapies to insulin was reviewed. In general, education strategies were very effective in teaching patients the importance of recognition and quick treatment of hypoglycaemia and more recently newer technologies with regard to continuous glucose monitoring systems have been used to supplement this. However the main limitations of these methods are that they require significant input from health care professionals. Reviewing adjunct drug strategies currently reported so far, shows that there seems to be a dissociation between symptomatic improvement during hypoglycaemia and improvements in the counter-regulatory hormonal responses, with none of the studies showing an improvement in both of these parameters. Therefore, one of our main aims in this thesis was to explore adjunct therapies to insulin to determine whether we could reduce frequency of hypoglycaemia and hence improve the magnitude of the counter-regulatory responses during insulin-induced hypoglycaemia (IIH).

We also explored whether there would be any improvements in the symptomatic and cognitive responses to IHH.

We aimed to look at existing licensed drugs to determine whether they would complement intensive insulin therapies. Prior to this, I went onto review aspects of glucose sensing, concentrating on the mechanisms present in the hypothalamus and in particular how this generates the counter-regulatory responses during hypoglycaemia. Various components of this complex cascade were reviewed in particular focusing on the ATP-sensitive potassium channels due to its relevance in STUDY 1. Research in cell culture and animal models has revealed that ATP-sensitive potassium (K_{ATP}) channels are integral to hypoglycaemia detection and initiation of CRRs, however, to date this has not been confirmed in human subjects. In STUDY 1, we examined whether K_{ATP} -channel openers (KCO) were able to amplify the CRR to hypoglycaemia in T1D subjects with IAH. A randomized double-blind placebo-controlled cross-over trial using a stepped hyperinsulinemic (1.5mu/kg/min) hypoglycaemia clamp (4.0, 3.5, 3.0, 2.5mmol/L) was performed in 12 T1D with prior ingestion of Diazoxide (D) (7mg/kg) (non-selective KCO) or placebo (P). Following Diazoxide T1D subjects showed a significant increase in adrenaline (ng/ml) responses at 2.5 mM glucose ($0.40 \pm (SE) 0.06$ vs. 0.29 ± 0.05 , D vs. P: $p < 0.05$) as well as a significant increase in noradrenaline(ng/ml) (0.85 ± 0.07 vs. 0.59 ± 0.06 , D vs. P: $p < 0.05$. Total symptoms of hypoglycaemia score were higher in the diazoxide group at 2.5mmol/L, (D; 21.5 and P; 19.3), $p = NS$. (Aut scores D; 10.25 ± 1.5 P; 8.9 ± 1.4). Although battery of cognitive tests showed shorter timings and possible cognitive preservation with diazoxide, none reached statistical significance. No adverse effects including significant hypotension were noted.

Therefore, diazoxide amplified the CRR hormonal but not symptomatic response to hypoglycaemia in subjects with long-duration T1D and IAH. This study shows for the first

time that K_{ATP} channels may play an important role in the detection of hypoglycaemia and that a therapeutic strategy using KCOs may offer a viable approach to restoring hypoglycaemia awareness in T1D. In addition, a subgroup analysis revealed that participants with E23K polymorphism in the K_{ATP} channel had a blunted response to oral diazoxide. This study has therefore shown for the first time the potential utility of K_{ATP} channel activators to improve counterregulatory responses to hypoglycaemia in individuals with T1D, and moreover that it may be possible to stratify therapeutic approaches by genotype.

The ability to choose patients by genotype and tailor drug therapies and/or dose is a very exciting prospect. We plan to conduct further studies whereby, for example we use a higher dose of K_{ATP} channel openers in patients who have the E23K polymorphism to determine if we can improve response. The risk of side effects with diazoxide at higher doses will need to be explored by further dose finding studies, to utilize the maximum safe dose. The other possibility is the development of a specific Kir6.2/SUR1 opener, which would theoretically eliminate the side effects seen by a compound such as diazoxide with more general systemic effects.

Therapies targeted at secondary physiological abnormalities in type 1 diabetes such as dysregulated glucagon secretion may also offer an effective and safe means of improving glycemic control. STUDY 2, examined the hypothesis that Dipeptidyl DiPeptidase 4 inhibition (DPP-4 i) in type 1 diabetes would result in improved glycemic control, reduced glucose variability and enhanced counterregulatory responses to hypoglycaemia.

Following informed consent, we recruited 14 subjects with moderately well controlled ($HbA1c\ 64\pm2$ mmol/mol) type 1 diabetes of long-standing (20.5 ± 2.7 years). Subjects received 12-weeks therapy with oral saxagliptin (5mg) or placebo. $HbA1c$, weight, total daily insulin dose, glucose-variability assessed via continuous glucose monitoring,

hypoglycaemia awareness, and symptomatic, cognitive and counterregulatory hormone responses to hypoglycaemia

We found no significant improvement in HbA1c (placebo vs. saxagliptin: 66 ± 2 vs 65 ± 2 mmol/mol, $p=0.5$), weight (74.6 ± 3.6 vs. 74.2 ± 3.3 kg, $p=0.64$), Low Blood Glucose Index, Average Daily Risk Range, and counterregulatory hormonal responses (AUC adrenaline, 25,775 vs. 24,454, placebo vs. saxagliptin, respectively, $p=0.76$) was seen following saxagliptin therapy in addition to usual insulin for 12-weeks. Our findings do not support the use of DPP-4 i in the management of type 1 diabetes.

The importance of dysregulated pancreatic α cell function particularly in the post-prandial state is increasingly appreciated. Although STUDY 2 did not show a significant benefit on the parameters measured, agents that have a more potent effects on the α -cell such as Amylin analogues or GLP-1 receptor agonists may prove more effective forms of adjunct therapy. There are currently on-going studies designed to address this question. It is notable that, as with DPP-4 i, GLP-1 infusion in subjects with c-peptide positive T1D has a far more profound effect on post-prandial glucose excursions and glucagon than is seen in c-peptide negative T1D and it has also been suggested that a major contribution to reduced post-prandial glucose excursions with GLP-1 in T1D reflect delayed gastric emptying as much as it does α -cell suppression. Overall, the case for α -cell targeted non-insulin adjunct therapy in T1D remains to be convincingly demonstrated. I would be keen to explore the current GLP-1 therapies, of which there are an increasing number of new compounds through physiological studies (such as mixed meal studies), to determine the best drug to lower post-prandial glucagon and therefore glucose excursion, and then potentially through reduction of hypoglycaemia, improve the counter-regulatory responses to further hypoglycaemia. I would then anticipate testing this agent as an adjunct therapy to IIT in further long term trials looking at similar parameters to STUDY 2.

Following STUDY 2, the direction of the thesis becomes broader with an exploration of the impact of glucose variability (GV) in patients with T1D. Several indices of GV have been measured over the last few decades, however to date, there is little consensus on which index to use, which may give some standardization of reporting particularly in clinical trials aiming at impacting GV.

There is also much debate and little evidence that GV in patients' with T1D has any significant long term effect. We however sought to explore the impact of GV and recurrent hypoglycaemia on the hypothalamo-pituitary adrenal axis (HPA), an area which is currently poor in evidence. Our main index of assessing this axis was a thorough analysis of the diurnal cortisol curve and its various components.

STUDY 3 particularly examined the relationship of recurrent hypoglycaemia through measurement of LBGi (Low Blood Glucose Index), an index of hypoglycaemia stress (through utilization of CGM) and the cortisol diurnal curve (salivary sampling). In this cross sectional study of subjects with T1D, there was a significant blunting (both in early and late decline) of the cortisol slope in those with a heavy burden of hypoglycaemia (as determined by a high LBGi). This was associated with a significantly increased AUC between 3-12 hours after wakening in this cohort. Therefore there appears to be prolonged exposure of endogenous steroids to the individual who is exposed to recurrent hypoglycaemia. Clues to the potential impact of this supraphysiological exposure to steroids can be derived from those with hypercortisolaemic conditions such as Cushing's syndrome. What is interesting is the similarities in the end effects of Cushing's and patients with T1D, in the propensity to develop cardiovascular risk factors such as hyperlipidemia and hypertension and development of mood disorders such as depression.

I would value the opportunity to explore this in greater detail. The challenge would be to disentangle the multiple risk factors in the development of these complications to determine the contribution of this mild hypercortisolaemic state on the physiological systems. Studies looking at different cohorts of patients who have similar abnormalities in their HPA system, such that they have exposure to supra-physiological but not pathological amounts of cortisol could be illuminating. Furthermore, recently, analysis of hair cortisol has been shown to be reflective of cortisol exposure over the last 3 months, and therefore, may provide a more robust measure which is unaffected by transient stresses of everyday life and therefore, could be used during further studies assessing the HPA axis.

With regard to GV assessment, there has been much debate as to the optimal time of CGM, in order for a thorough evaluation of GV. Naturally as CGM duration increases, there is a reduction in %error from the 90 day value (gold standard), but reached a median value of <10% error at 12 days, suggesting that this might be an optimal time period. Recently, Abbott has launched a Freestyle Libre Flash Glucose monitoring System which has the ability to monitor for 14 days with one sensor, and has the ability to record and store 90 days of CGM data. This new technology with its longer monitoring period, may therefore provide a more robust measurement of GV, than the technology used during my studies.

Further study using a biochemical marker such as 1,5 anhydroglucitol (structurally similar to glucose, and has been shown to be inversely correlated with hyperglycaemia) has been shown to be well correlated with several markers of GV (SD, MAGE and LI), however, this correlation only seemed to exist in patients with moderate glycaemic control (mean CGMS glucose < 10mmol/L(Seok, Huh et al. 2015) as opposed to those with poor glycaemic control. The other added benefit, is that it is thought to relate very well to GV over the preceding few weeks, avoiding the need to attach a patient to a needle for several days. Therefore this

technique may have been less influenced by patient's behavior (with a change often seen when patients are attached to a device).

With the disturbances in cortisol patterns seen in those with T1D, we also went on to study whether we could ascertain the exact location of the underlying problem within the HPA system in STUDY 4, by conducting CRH testing in a cohort of subjects from STUDY 3.

This showed that there was a trend ($p=0.08$) towards a lower ACTH response in T1D compared to controls, with no difference in cortisol responses to CRH stimulation. This suggests that there may be a pituitary insensitivity as a result of continuous negative feedback from increased levels of endogenous steroids in those with recurrent hypoglycaemia. If there is indeed a confirmed, real dysregulation of the HPA system, in the future, I would anticipate using smaller doses of CRH to determine the true sensitivity of the pituitary gland and potentially, a larger dose may be required to determine the sensitivity of the adrenal glands, but currently based on the current study, we don't have any evidence that the adrenal glands are not sensitive to the effects of ACTH.

Ultimately, we would want to determine whether improvement of glycaemic parameters, particularly reduction in frequency of hypoglycaemia, would be able to reverse the dysregulation of the HPA system, if one indeed is present, and reduce the burden of endogenous steroids on the physiological systems, and therefore improve long term outcomes for patients particularly with regard to macrovascular complications of T1D.

This thesis has really enabled me to understand some of the key problems faced by patients with T1D such as recurrent hypoglycaemia (RH) and glycaemic variability (GV) and its wider impact on other physiological systems. Many trials have focused on HbA1c, which is clearly related to long term micro and macrovascular complications, however both RH and GV have significant short term morbidity and has a significant impact on patient's immediate

quality of life, and therefore needs to be addressed and hopefully I have shown in this body of work, different therapeutic strategies that should be considered in addressing the latter. The impact of GV has always been debated in patients' with T1D, but we show that GV and recurrent hypoglycaemia can potentially cause a disturbance in the HPA axis and ultimately affect long term outcomes such as macrovascular disease.

Original papers and abstracts

1. PS George, RJ McCrimmon. Saxagliptin co-therapy in c-peptide negative Type 1 diabetes does not improve counterregulatory responses to hypoglycaemia. *Diabetes Medicine*. 2015; epub
2. PS George, R Tavendale, CAN Palmer, RJ McCrimmon. Diazoxide improves hormonal counterregulatory responses to acute hypoglycemia in long-standing Type 1 Diabetes. *Diabetes*. 2015; 64 :2234-2241
3. George PS, Mackie A, Connell J, McCrimmon RJ. Conference Report; Glycaemic variability and the cortisol curve in *Endocrine Abstracts (34)* 24-27 March 2014. Liverpool
4. BES 2014. Glycaemic variability and the cortisol curve. P George, A Mackie, J Connell, R McCrimmon
5. PS George, RJ McCrimmon. Conference Report; Diazoxide improves neuroendocrine responses in those with Type 1 Diabetes in *Diabetes (62) Supp 1*. 21-25th June 2013. Chicago

Reviews

6. PS George, RJ McCrimmon. Therapeutic strategies to prevent hypoglycaemia using pharmacological interventions. *Diabetic Hypoglycaemia*. Oct 2012, 5(2) 13-16.
7. PS George, RJ McCrimmon. Potential Role on Non-Insulin Adjunct therapy in Type 1 Diabetes. *Diabetic Med* 2012. Jul 18.
8. Drug Note. Diazoxide. *Practical Diabetes* 2012; 29(1) 36-37 PS George, RJ McCrimmon

Prizes

9. Awarded the Fitzgerald Peel Prize at the Scottish Society of Physicians for 2014 Sept; Diazoxide improves the neuroendocrine responses in Acute Insulin Induced Hypoglycaemia in patients with Type 1 Diabetes
10. Awarded the Young Investigator Travel Grant at the ADA 2013 Chicago for Diazoxide improves the neuroendocrine responses in Acute Insulin Induced Hypoglycaemia in patients with Type 1 Diabetes

References

- (1971). "Diazoxide: a review of its pharmacological properties and therapeutic use in hypertensive crises." Drugs **2**(2): 78-137.
- (1993). "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group." The New England journal of medicine **329**(14): 977-986.
- (1995). "The relationship of glycemic exposure (HbA1c) to the risk of development and progression of retinopathy in the diabetes control and complications trial." Diabetes **44**(8): 968-983.
- (1997). "Hypoglycemia in the Diabetes Control and Complications Trial. The Diabetes Control and Complications Trial Research Group." Diabetes **46**(2): 271-286.
- (1997). "Wechsler Memory Scale—Third Edition." The Psychological Corporation.
- (2008). "The accuracy of the Guardian RT continuous glucose monitor in children with type 1 diabetes." Diabetes technology & therapeutics **10**(4): 266-272.
- (!!! INVALID CITATION !!!).
- Abe, K., J. Kroning, M. A. Greer and V. Critchlow (1979). "Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin." Neuroendocrinology **29**(2): 119-131.
- Abercrombie, H. C., J. Giese-Davis, S. Sephton, E. S. Epel, J. M. Turner-Cobb and D. Spiegel (2004). "Flattened cortisol rhythms in metastatic breast cancer patients." Psychoneuroendocrinology **29**(8): 1082-1092.
- Adam, E. K., L. C. Hawkey, B. M. Kudielka and J. T. Cacioppo (2006). "Day-to-day dynamics of experience--cortisol associations in a population-based sample of older adults." Proceedings of the National Academy of Sciences of the United States of America **103**(45): 17058-17063.
- Aguilar-Bryan, L. and J. Bryan (1999). "Molecular biology of adenosine triphosphate-sensitive potassium channels." Endocrine reviews **20**(2): 101-135.
- Aguilar-Bryan, L., J. Bryan and M. Nakazaki (2001). "Of mice and men: K(ATP) channels and insulin secretion." Recent Prog Horm Res **56**: 47-68.
- Ahren, B. (2009). "Clinical results of treating type 2 diabetic patients with sitagliptin, vildagliptin or saxagliptin--diabetes control and potential adverse events." Best practice & research. Clinical endocrinology & metabolism **23**(4): 487-498.
- Ahren, B., A. Schweizer, S. Dejager, E. B. Villhauer, B. E. Dunning and J. E. Foley (2011). "Mechanisms of action of the dipeptidyl peptidase-4 inhibitor vildagliptin in humans." Diabetes Obes Metab **13**(9): 775-783.
- Aizawa, T., N. Yasuda and M. A. Greer (1981). "Hypoglycemia stimulates ACTH secretion through a direct effect on the basal hypothalamus." Metabolism: clinical and experimental **30**(10): 996-1000.
- Alexander, A. (2003). "Elevated salivary cortisol due to sleep deprivation." Occupational medicine **53**(6): 409; author reply 410.
- Alghothani, N. and K. M. Dungan (2011). "The effect of glycemic variability on counterregulatory hormone responses to hypoglycemia in young children and adolescents with type 1 diabetes." Diabetes Technol Ther **13**(11): 1085-1089.
- Amiel, S. A., R. S. Sherwin, D. C. Simonson and W. V. Tamborlane (1988). "Effect of intensive insulin therapy on glycemic thresholds for counterregulatory hormone release." Diabetes **37**(7): 901-907.
- Amiel, S. A., D. C. Simonson, R. S. Sherwin, A. A. Lauritano and W. V. Tamborlane (1987). "Exaggerated epinephrine responses to hypoglycemia in normal and insulin-dependent diabetic children." The Journal of pediatrics **110**(6): 832-837.
- Amiel, S. A., D. C. Simonson, W. V. Tamborlane, R. A. DeFronzo and R. S. Sherwin (1987). "Rate of glucose fall does not affect counterregulatory hormone responses to hypoglycemia in normal and diabetic humans." Diabetes **36**(4): 518-522.

Andreis, P. G., G. Neri and G. G. Nussdorfer (1991). "Corticotropin-releasing hormone (CRH) directly stimulates corticosterone secretion by the rat adrenal gland." Endocrinology **128**(2): 1198-1200.

Andrews, R. C., O. Herlihy, D. E. Livingstone, R. Andrew and B. R. Walker (2002). "Abnormal cortisol metabolism and tissue sensitivity to cortisol in patients with glucose intolerance." The Journal of clinical endocrinology and metabolism **87**(12): 5587-5593.

Andrews, R. V. and G. E. Folk, Jr. (1964). "Circadian Metabolic Patterns in Cultured Hamster Adrenal Glands." Comp Biochem Physiol **11**: 393-409.

Araki, R., H. Takahashi, R. Fukumura, F. Sun, N. Umeda, M. Sujino, S. T. Inouye, T. Saito and M. Abe (2004). "Restricted expression and photic induction of a novel mouse regulatory factor X4 transcript in the suprachiasmatic nucleus." The Journal of biological chemistry **279**(11): 10237-10242.

Arbelaez, A. M., W. J. Powers, T. O. Videen, J. L. Price and P. E. Cryer (2008). "Attenuation of counterregulatory responses to recurrent hypoglycemia by active thalamic inhibition: a mechanism for hypoglycemia-associated autonomic failure." Diabetes **57**(2): 470-475.

Arsenault-Lapierre, G., H. Chertkow and S. Lupien (2010). "Seasonal effects on cortisol secretion in normal aging, mild cognitive impairment and Alzheimer's disease." Neurobiology of aging **31**(6): 1051-1054.

Ashcroft, S. J. and F. M. Ashcroft (1990). "Properties and functions of ATP-sensitive K-channels." Cellular signalling **2**(3): 197-214.

Ashford, M. L., P. R. Boden and J. M. Treherne (1990). "Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K⁺ channels." Pflugers Archiv - European Journal of Physiology **415**(4): 479-483.

Ashford, M. L., P. R. Boden and J. M. Treherne (1990). "Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K⁺ channels." Pflugers Archiv : European journal of physiology **415**(4): 479-483.

Assef, S. J., R. L. Lennon, K. A. Jones, M. J. Burke and T. L. Behrens (1993). "A versatile, computer-controlled, closed-loop system for continuous infusion of muscle relaxants." Mayo Clinic proceedings. Mayo Clinic **68**(11): 1074-1080.

Axelrod, J. and T. D. Reisine (1984). "Stress hormones: their interaction and regulation." Science **224**(4648): 452-459.

Axelsen, M., C. Wesslau, P. Lonnroth, R. Arvidsson Lenner and U. Smith (1999). "Bedtime uncooked cornstarch supplement prevents nocturnal hypoglycaemia in intensively treated type 1 diabetes subjects." Journal of internal medicine **245**(3): 229-236.

Badrick, E., M. Bobak, A. Britton, C. Kirschbaum, M. Marmot and M. Kumari (2008). "The relationship between alcohol consumption and cortisol secretion in an aging cohort." The Journal of clinical endocrinology and metabolism **93**(3): 750-757.

Baghurst, P. A., D. Rodbard and F. J. Cameron (2010). "The minimum frequency of glucose measurements from which glycemic variation can be consistently assessed." Journal of diabetes science and technology **4**(6): 1382-1385.

Baid, S. and L. K. Nieman (2004). "Glucocorticoid excess and hypertension." Current hypertension reports **6**(6): 493-499.

Bale, T. L. and W. W. Vale (2004). "CRF and CRF receptors: role in stress responsivity and other behaviors." Annu Rev Pharmacol Toxicol **44**: 525-557.

Balsalobre, A., S. A. Brown, L. Marcacci, F. Tronche, C. Kellendonk, H. M. Reichardt, G. Schutz and U. Schibler (2000). "Resetting of circadian time in peripheral tissues by glucocorticoid signaling." Science **289**(5488): 2344-2347.

Balu, D. T. and I. Lucki (2009). "Adult hippocampal neurogenesis: regulation, functional implications, and contribution to disease pathology." Neuroscience and biobehavioral reviews **33**(3): 232-252.

Banting, F. G., C. H. Best, J. B. Collip, W. R. Campbell and A. A. Fletcher (1922). "Pancreatic Extracts in the Treatment of Diabetes Mellitus." Canadian Medical Association journal **12**(3): 141-146.

Barnett, R. C., A. Steptoe and K. C. Gareis (2005). "Marital-role quality and stress-related psychobiological indicators." Annals of behavioral medicine : a publication of the Society of Behavioral Medicine **30**(1): 36-43.

Basu, A., S. Dube, M. Slama, I. Errazuriz, J. C. Amezcua, Y. C. Kudva, T. Peyser, R. E. Carter, C. Cobelli and R. Basu (2013). "Time lag of glucose from intravascular to interstitial compartment in humans." Diabetes **62**(12): 4083-4087.

Battelino, T., I. Conget, B. Olsen, I. Schutz-Fuhrmann, E. Hommel, R. Hoogma, U. Schierloh, N. Sulli and J. Bolinder (2012). "The use and efficacy of continuous glucose monitoring in type 1 diabetes treated with insulin pump therapy: a randomised controlled trial." Diabetologia **55**(12): 3155-3162.

Baylis, P. H., R. L. Zerbe and G. L. Robertson (1981). "Arginine vasopressin response to insulin-induced hypoglycemia in man." J Clin Endocrinol Metab **53**(5): 935-940.

Beall, C., D. L. Hamilton, J. Gallagher, L. Logie, K. Wright, M. P. Soutar, S. Dadak, F. B. Ashford, E. Haythorne, Q. Du, A. Jovanovic, R. J. McCrimmon and M. L. Ashford (2012). "Mouse hypothalamic GT1-7 cells demonstrate AMPK-dependent intrinsic glucose-sensing behaviour." Diabetologia **55**(9): 2432-2444.

Beck, R. W., I. B. Hirsch, L. Laffel, W. V. Tamborlane, B. W. Bode, B. Buckingham, P. Chase, R. Clemons, R. Fiallo-Scharer, L. A. Fox, L. K. Gilliam, E. S. Huang, C. Kollman, A. J. Kowalski, J. M. Lawrence, J. Lee, N. Murras, M. O'Grady, K. J. Ruedy, M. Tansey, E. Tsalikian, S. A. Weinzimer, D. M. Wilson, H. Wolpert, T. Wysocki and D. Xing (2009). "The effect of continuous glucose monitoring in well-controlled type 1 diabetes." Diabetes care **32**(8): 1378-1383.

Beech, D. J., H. Zhang, K. Nakao and T. B. Bolton (1993). "K channel activation by nucleotide diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells." British journal of pharmacology **110**(2): 573-582.

Benzo, C. A. (1983). "Minireview. The hypothalamus and blood glucose regulation." Life sciences **32**(22): 2509-2515.

Bergman, R. N., D. T. Finegood and M. Ader (1985). "Assessment of insulin sensitivity in vivo." Endocr Rev **6**(1): 45-86.

Berlin, I., A. Grimaldi, F. Thervet and A. J. Puech (1987). "Hypoglycaemia unawareness." Lancet **2**(8565): 966.

Beverly, J. L., M. G. De Vries, S. D. Bouman and L. M. Arseneau (2001). "Noradrenergic and GABAergic systems in the medial hypothalamus are activated during hypoglycemia." American journal of physiology. Regulatory, integrative and comparative physiology **280**(2): R563-569.

Bhattacharyya, M. R., G. J. Molloy and A. Steptoe (2008). "Depression is associated with flatter cortisol rhythms in patients with coronary artery disease." Journal of psychosomatic research **65**(2): 107-113.

Bingham, E., D. Hopkins, A. Pernet, H. Reid, I. A. Macdonald and S. A. Amiel (2003). "The effects of KATP channel modulators on counterregulatory responses and cognitive function during acute controlled hypoglycaemia in healthy men: a pilot study." Diabetic medicine : a journal of the British Diabetic Association **20**(3): 231-237.

Bingham, E., D. Hopkins, A. Pernet, H. Reid, I. A. Macdonald and S. A. Amiel (2003). "The effects of KATP channel modulators on counterregulatory responses and cognitive function during acute controlled hypoglycaemia in healthy men: a pilot study." Diabet Med **20**(3): 231-237.

Bingham, E. M., J. T. Dunn, D. Smith, J. Sutcliffe-Goulden, L. J. Reed, P. K. Marsden and S. A. Amiel (2005). "Differential changes in brain glucose metabolism during hypoglycaemia accompany loss of hypoglycaemia awareness in men with type 1 diabetes mellitus. An [11C]-3-O-methyl-D-glucose PET study." Diabetologia **48**(10): 2080-2089.

Bitar, M. S. (1998). "Glucocorticoid dynamics and impaired wound healing in diabetes mellitus." The American journal of pathology **152**(2): 547-554.

Bjork, E., C. Berne, O. Kampe, L. Wibell, P. Oskarsson and F. A. Karlsson (1996). "Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes." Diabetes **45**(10): 1427-1430.

Bode, B., R. W. Beck, D. Xing, L. Gilliam, I. Hirsch, C. Kollman, L. Laffel, K. J. Ruedy, W. V. Tamborlane, S. Weinzimer and H. Wolpert (2009). "Sustained benefit of continuous glucose monitoring on A1C, glucose profiles, and hypoglycemia in adults with type 1 diabetes." Diabetes care **32**(11): 2047-2049.

Bohme, P., E. Bertin, E. Cosson and N. Chevalier (2013). "Fear of hypoglycaemia in patients with type 1 diabetes: Do patients and diabetologists feel the same way?" Diabetes & metabolism **39**(1): 63-70.

Bolo, N. R., G. Musen, A. M. Jacobson, K. Weinger, R. L. McCartney, V. Flores, P. F. Renshaw and D. C. Simonson (2011). "Brain activation during working memory is altered in patients with type 1 diabetes during hypoglycemia." Diabetes **60**(12): 3256-3264.

Bonfanti, D. H., L. P. Alcazar, P. A. Arakaki, L. T. Martins, B. C. Agustini, F. G. de Moraes Rego and H. R. Frigeri (2015). "ATP-dependent potassium channels and type 2 diabetes mellitus." Clin Biochem **48**(7-8): 476-482.

Borg, M. A., W. P. Borg, W. V. Tamborlane, M. L. Brines, G. I. Shulman and R. S. Sherwin (1999). "Chronic hypoglycemia and diabetes impair counterregulation induced by localized 2-deoxy-glucose perfusion of the ventromedial hypothalamus in rats." Diabetes **48**(3): 584-587.

Borg, M. A., R. S. Sherwin, W. P. Borg, W. V. Tamborlane and G. I. Shulman (1997). "Local ventromedial hypothalamus glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats." The Journal of clinical investigation **99**(2): 361-365.

Borg, W. P., M. J. During, R. S. Sherwin, M. A. Borg, M. L. Brines and G. I. Shulman (1994). "Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia." The Journal of clinical investigation **93**(4): 1677-1682.

Borg, W. P., R. S. Sherwin, M. J. During, M. A. Borg and G. I. Shulman (1995). "Local ventromedial hypothalamus glucopenia triggers counterregulatory hormone release." Diabetes **44**(2): 180-184.

Born, J., E. Spath-Schwalbe, H. Schwachenhofer, W. Kern and H. L. Fehm (1989). "Influences of corticotropin-releasing hormone, adrenocorticotropin, and cortisol on sleep in normal man." The Journal of clinical endocrinology and metabolism **68**(5): 904-911.

Bornstein, S. R., M. Breidert, M. Ehrhart-Bornstein, B. Kloos and W. A. Scherbaum (1995). "Plasma catecholamines in patients with Addison's disease." Clinical endocrinology **42**(2): 215-218.

Boyle, P. J., S. F. Kempers, A. M. O'Connor and R. J. Nagy (1995). "Brain glucose uptake and unawareness of hypoglycemia in patients with insulin-dependent diabetes mellitus." The New England journal of medicine **333**(26): 1726-1731.

Boyle, P. J., S. F. Kempers, A. M. O'Connor and R. J. Nagy (1995). "Brain glucose uptake and unawareness of hypoglycemia in patients with insulin-dependent diabetes mellitus.[see comment]." New England Journal of Medicine **333**(26): 1726-1731.

Boyle, P. J., N. S. Schwartz, S. D. Shah, W. E. Clutter and P. E. Cryer (1988). "Plasma glucose concentrations at the onset of hypoglycemic symptoms in patients with poorly controlled diabetes and in nondiabetics." The New England journal of medicine **318**(23): 1487-1492.

Bragd, J., U. Adamson, L. B. Backlund, P. E. Lins, E. Moberg and P. Oskarsson (2008). "Can glycaemic variability, as calculated from blood glucose self-monitoring, predict the development of complications in type 1 diabetes over a decade?" Diabetes & metabolism **34**(6 Pt 1): 612-616.

Brands, A. M., G. J. Biessels, E. H. de Haan, L. J. Kappelle and R. P. Kessels (2005). "The effects of type 1 diabetes on cognitive performance: a meta-analysis." Diabetes care **28**(3): 726-735.

Brierley, E. J., D. L. Broughton, O. F. James and K. G. Alberti (1995). "Reduced awareness of hypoglycaemia in the elderly despite an intact counter-regulatory response." QJM : monthly journal of the Association of Physicians **88**(6): 439-445.

Briscoe, V. J., A. C. Ertl, D. B. Tate and S. N. Davis (2008). "Effects of the selective serotonin reuptake inhibitor fluoxetine on counterregulatory responses to hypoglycemia in individuals with type 1 diabetes." Diabetes **57**(12): 3315-3322.

Brod, M., T. Christensen, T. L. Thomsen and D. M. Bushnell (2011). "The impact of non-severe hypoglycemic events on work productivity and diabetes management." Value Health **14**(5): 665-671.

Brownlee, M. (2005). "The pathobiology of diabetic complications: a unifying mechanism." Diabetes **54**(6): 1615-1625.

Brownlee, M. and I. B. Hirsch (2006). "Glycemic variability: a hemoglobin A1c-independent risk factor for diabetic complications." JAMA : the journal of the American Medical Association **295**(14): 1707-1708.

Buckingham, B., J. Block, J. Burdick, A. Kalajian, C. Kollman, M. Choy, D. M. Wilson and P. Chase (2005). "Response to nocturnal alarms using a real-time glucose sensor." Diabetes technology & therapeutics **7**(3): 440-447.

bugler, J. (2008). " An estimation of the amount of data required to measure glycaemic variability." Adv Technol Treatments Diabetes **88**.

Bulsara, M. K., C. D. Holman, E. A. Davis and T. W. Jones (2004). "The impact of a decade of changing treatment on rates of severe hypoglycemia in a population-based cohort of children with type 1 diabetes." Diabetes care **27**(10): 2293-2298.

Cameron, F. J., S. M. Donath and P. A. Baghurst (2010). "Measuring glycaemic variation." Current diabetes reviews **6**(1): 17-26.

Cameron, O. G., Z. Kronfol, J. F. Greden and B. J. Carroll (1984). "Hypothalamic-pituitary-adrenocortical activity in patients with diabetes mellitus." Archives of general psychiatry **41**(11): 1090-1095.

Caprio, S., G. Gerety, W. V. Tamborlane, T. Jones, M. Diamond, R. Jacob and R. S. Sherwin (1991). "Opiate blockade enhances hypoglycemic counterregulation in normal and insulin-dependent diabetic subjects." The American journal of physiology **260**(6 Pt 1): E852-858.

Caprio, S., W. V. Tamborlane, K. Zych, K. Gerow and R. S. Sherwin (1993). "Loss of potentiating effect of hypoglycemia on the glucagon response to hyperaminoacidemia in IDDM." Diabetes **42**(4): 550-555.

Carnes, M., S. J. Lent, B. Goodman, C. Mueller, J. Saydoff and S. Erisman (1990). "Effects of immunoneutralization of corticotropin-releasing hormone on ultradian rhythms of plasma adrenocorticotropin." Endocrinology **126**(4): 1904-1913.

Carson, K. A., J. S. Hanker and N. Kirshner (1982). "The adrenal medulla of the diabetic mouse (C57BL/KsJ, db/db): biochemical and morphological changes." Comparative biochemistry and physiology. A, Comparative physiology **72**(2): 279-285.

Cengiz, E. and W. V. Tamborlane (2009). "A tale of two compartments: interstitial versus blood glucose monitoring." Diabetes Technol Ther **11 Suppl 1**: S11-16.

Ceriello, A. (2005). "Postprandial hyperglycemia and diabetes complications: is it time to treat?" Diabetes **54**(1): 1-7.

Ceriello, A. and M. A. Ihnat (2010). "'Glycaemic variability': a new therapeutic challenge in diabetes and the critical care setting." Diabetic medicine : a journal of the British Diabetic Association **27**(8): 862-867.

Ceriello, A., L. La Sala, V. De Nigris, G. Pujadas, M. Rondinelli and S. Genovese (2015). "GLP-1 reduces metalloproteinase-9 induced by both hyperglycemia and hypoglycemia in type 1 diabetes. The possible role of oxidative stress." Therapeutics and clinical risk management **11**: 901-903.

Ceriello, A., A. Novials, E. Ortega, L. La Sala, G. Pujadas, R. Testa, A. R. Bonfigli, K. Esposito and D. Giugliano (2012). "Evidence that hyperglycemia after recovery from hypoglycemia worsens endothelial function and increases oxidative stress and inflammation in healthy control subjects and subjects with type 1 diabetes." Diabetes **61**(11): 2993-2997.

Ceriello, A., A. Novials, E. Ortega, G. Pujadas, L. La Sala, R. Testa, A. R. Bonfigli and S. Genovese (2014). "Hyperglycemia following recovery from hypoglycemia worsens endothelial damage and thrombosis activation in type 1 diabetes and in healthy controls." Nutrition, metabolism, and cardiovascular diseases : NMCD **24**(2): 116-123.

Champaneri, S., G. S. Wand, S. S. Malhotra, S. S. Casagrande and S. H. Golden (2010). "Biological basis of depression in adults with diabetes." Current diabetes reports **10**(6): 396-405.

Champaneri, S., X. Xu, M. R. Carnethon, A. G. Bertoni, T. Seeman, A. S. DeSantis, A. Diez Roux, S. Shrager and S. H. Golden (2013). "Diurnal salivary cortisol is associated with body mass index and waist circumference: the Multiethnic Study of Atherosclerosis." Obesity (Silver Spring) **21**(1): E56-63.

Chan, O., S. Chan, K. Inouye, K. Shum, S. G. Matthews and M. Vranic (2002). "Diabetes impairs hypothalamo-pituitary-adrenal (HPA) responses to hypoglycemia, and insulin treatment normalizes HPA but not epinephrine responses." Diabetes **51**(6): 1681-1689.

Chan, O., S. Chan, K. Inouye, M. Vranic and S. G. Matthews (2001). "Molecular regulation of the hypothalamo-pituitary-adrenal axis in streptozotocin-induced diabetes: effects of insulin treatment." Endocrinology **142**(11): 4872-4879.

Chan, O., H. Cheng, R. Herzog, D. Czyzyk, W. Zhu, A. Wang, R. J. McCrimmon, M. R. Seashore and R. S. Sherwin (2008). "Increased GABAergic tone in the ventromedial hypothalamus contributes to suppression of counterregulatory responses after antecedent hypoglycemia." Diabetes **57**(5): 1363-1370.

Chan, O., K. Inouye, M. Vranic and S. G. Matthews (2002). "Hyperactivation of the hypothalamo-pituitary-adrenocortical axis in streptozotocin-diabetes is associated with reduced stress responsiveness and decreased pituitary and adrenal sensitivity." Endocrinology **143**(5): 1761-1768.

Chase, H. P., K. Lutz, R. Pencek, B. Zhang and L. Porter (2009). "Pramlintide lowered glucose excursions and was well-tolerated in adolescents with type 1 diabetes: results from a randomized, single-blind, placebo-controlled, crossover study." The Journal of pediatrics **155**(3): 369-373.

Chen, J., F. M. Couto, A. H. Minn and A. Shalev (2006). "Exenatide inhibits beta-cell apoptosis by decreasing thioredoxin-interacting protein." Biochemical and biophysical research communications **346**(3): 1067-1074.

Choudhary, C., Davies, D, Freeman, J. Emery, C. Heller, S (2006). Determining the ability of CGMS to detect clinically relevant hypoglycaemia. ADA.

Choudhary, P., J. Geddes, J. V. Freeman, C. J. Emery, S. R. Heller and B. M. Frier (2010). "Frequency of biochemical hypoglycaemia in adults with Type 1 diabetes with and without impaired awareness of hypoglycaemia: no identifiable differences using continuous glucose monitoring." Diabet Med **27**(6): 666-672.

Choudhary, P., K. Lonnen, C. J. Emery, J. V. Freeman, K. M. McLeod and S. R. Heller (2011). "Relationship between interstitial and blood glucose during hypoglycemia in subjects with type 2 diabetes." Diabetes technology & therapeutics **13**(11): 1121-1127.

Choudhary, P., M. R. Rickels, P. A. Senior, M. C. Vantyghem, P. Maffi, T. W. Kay, B. Keymeulen, N. Inagaki, F. Saudek, R. Lehmann and B. J. Hering (2015). "Evidence-informed clinical practice recommendations for treatment of type 1 diabetes complicated by problematic hypoglycemia." Diabetes Care **38**(6): 1016-1029.

Chrousos, G. P. and T. Kino (2007). "Glucocorticoid action networks and complex psychiatric and/or somatic disorders." Stress **10**(2): 213-219.

Chrousos, G. P., H. M. Schulte, E. H. Oldfield, P. W. Gold, G. B. Cutler, Jr. and D. L. Loriaux (1984). "The corticotropin-releasing factor stimulation test. An aid in the evaluation of patients with Cushing's syndrome." The New England journal of medicine **310**(10): 622-626.

Chung, S., G. H. Son and K. Kim (2011). "Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications." Biochimica et biophysica acta **1812**(5): 581-591.

Clarke, W. L., D. J. Cox, L. A. Gonder-Frederick, D. Julian, D. Schlundt and W. Polonsky (1995). "Reduced awareness of hypoglycemia in adults with IDDM. A prospective study of hypoglycemic frequency and associated symptoms." Diabetes care **18**(4): 517-522.

Clements, A. D. and C. R. Parker (1998). "The relationship between salivary cortisol concentrations in frozen versus mailed samples." Psychoneuroendocrinology **23**(6): 613-616.

Clore, J. N. and L. Thurby-Hay (2009). "Glucocorticoid-induced hyperglycemia." Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists **15**(5): 469-474.

Clow, A., F. Hucklebridge, T. Stalder, P. Evans and L. Thorn (2010). "The cortisol awakening response: more than a measure of HPA axis function." Neuroscience and biobehavioral reviews **35**(1): 97-103.

Cohen, S., E. Frank, W. J. Doyle, D. P. Skoner, B. S. Rabin and J. M. Gwaltney, Jr. (1998). "Types of stressors that increase susceptibility to the common cold in healthy adults." Health psychology :

official journal of the Division of Health Psychology, American Psychological Association **17**(3): 214-223.

Cohen, S., J. E. Schwartz, E. Epel, C. Kirschbaum, S. Sidney and T. Seeman (2006). "Socioeconomic status, race, and diurnal cortisol decline in the Coronary Artery Risk Development in Young Adults (CARDIA) Study." Psychosomatic medicine **68**(1): 41-50.

Coiro, V., R. Volpi, L. Capretti, G. Speroni, P. Caffarra, A. Scaglioni, L. Malvezzi, A. Castelli, G. Caffarri, G. Rossi and et al. (1995). "Low-dose ovine corticotropin-releasing hormone stimulation test in diabetes mellitus with or without neuropathy." Metabolism: clinical and experimental **44**(4): 538-542.

Cole, T. J., J. A. Blendy, A. P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker and G. Schutz (1995). "Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation." Genes & development **9**(13): 1608-1621.

Conway-Campbell, B. L., J. R. Pooley, G. L. Hager and S. L. Lightman (2012). "Molecular dynamics of ultradian glucocorticoid receptor action." Molecular and cellular endocrinology **348**(2): 383-393.

Conway, A. R., M. J. Kane, M. F. Bunting, D. Z. Hambrick, O. Wilhelm and R. W. Engle (2005). "Working memory span tasks: A methodological review and user's guide." Psychon Bull Rev **12**(5): 769-786.

Cook, D. L. and C. N. Hales (1984). "Intracellular ATP directly blocks K⁺ channels in pancreatic B-cells." Nature **311**(5983): 271-273.

Cook, W., B. Bryzinski, J. Slater, R. Frederich and E. Allen (2013). "Saxagliptin efficacy and safety in patients with type 2 diabetes mellitus and cardiovascular disease history or cardiovascular risk factors: results of a pooled analysis of phase 3 clinical trials." Postgraduate medicine **125**(3): 145-154.

Corrall, R. J., R. J. Webber and B. M. Frier (1980). "Increase in coagulation factor VIII activity in man following acute hypoglycaemia: mediation via an adrenergic mechanism." British journal of haematology **44**(2): 301-305.

Coste, J., G. Strauch, M. Letrait and X. Bertagna (1994). "Reliability of hormonal levels for assessing the hypothalamic-pituitary-adrenocortical system in clinical pharmacology." British journal of clinical pharmacology **38**(5): 474-479.

Cox, D., L. Gonder-Frederick, W. Polonsky, D. Schlundt, D. Julian and W. Clarke (1995). "A multicenter evaluation of blood glucose awareness training-II." Diabetes care **18**(4): 523-528.

Cox, D. J., L. Gonder-Frederick, B. Antoun, P. E. Cryer and W. L. Clarke (1993). "Perceived symptoms in the recognition of hypoglycemia." Diabetes care **16**(2): 519-527.

Cox, D. J., L. Gonder-Frederick, L. Ritterband, W. Clarke and B. P. Kovatchev (2007). "Prediction of severe hypoglycemia." Diabetes care **30**(6): 1370-1373.

Cox, D. J., A. Irvine, L. Gonder-Frederick, G. Nowacek and J. Butterfield (1987). "Fear of hypoglycemia: quantification, validation, and utilization." Diabetes care **10**(5): 617-621.

Cox, D. J., B. P. Kovatchev, D. M. Julian, L. A. Gonder-Frederick, W. H. Polonsky, D. G. Schlundt and W. L. Clarke (1994). "Frequency of severe hypoglycemia in insulin-dependent diabetes mellitus can be predicted from self-monitoring blood glucose data." J Clin Endocrinol Metab **79**(6): 1659-1662.

Cranston, I., J. Lomas, A. Maran, I. Macdonald and S. A. Amiel (1994). "Restoration of hypoglycaemia awareness in patients with long-duration insulin-dependent diabetes." Lancet **344**(8918): 283-287.

Cranston, I., L. J. Reed, P. K. Marsden and S. A. Amiel (2001). "Changes in regional brain (18)F-fluorodeoxyglucose uptake at hypoglycemia in type 1 diabetic men associated with hypoglycemia unawareness and counter-regulatory failure." Diabetes **50**(10): 2329-2336.

Creutzfeldt, W. O., N. Kleine, B. Willms, C. Orskov, J. J. Holst and M. A. Nauck (1996). "Glucagonostatic actions and reduction of fasting hyperglycemia by exogenous glucagon-like peptide I(7-36) amide in type I diabetic patients." Diabetes care **19**(6): 580-586.

Cryer, P. (2002). "Negotiating the Barrier of Hypoglycemia in Diabetes." Diabetes Spectrum **15**(1): 20-27.

- Cryer, P. E. (1984). "Catecholamines and metabolism. Introduction." The American journal of physiology **247**(1 Pt 1): E1-3.
- Cryer, P. E. (1994). "Banting Lecture. Hypoglycemia: the limiting factor in the management of IDDM." Diabetes **43**(11): 1378-1389.
- Cryer, P. E. (2002). "Hypoglycaemia: the limiting factor in the glycaemic management of Type I and Type II diabetes." Diabetologia **45**(7): 937-948.
- Cryer, P. E. (2007). "Hypoglycemia, functional brain failure, and brain death." J Clin Invest **117**(4): 868-870.
- Cryer, P. E. (2011). "Death during intensive glycemic therapy of diabetes: mechanisms and implications." Am J Med **124**(11): 993-996.
- Cryer, P. E., S. N. Davis and H. Shamoon (2003). "Hypoglycemia in diabetes." Diabetes Care **26**(6): 1902-1912.
- Cryer, P. E. and J. E. Gerich (1985). "Glucose counterregulation, hypoglycemia, and intensive insulin therapy in diabetes mellitus." The New England journal of medicine **313**(4): 232-241.
- Cummins, E., P. Royle, A. Snaith, A. Greene, L. Robertson, L. McIntyre and N. Waugh (2010). "Clinical effectiveness and cost-effectiveness of continuous subcutaneous insulin infusion for diabetes: systematic review and economic evaluation." Health technology assessment **14**(11): iii-iv, xi-xvi, 1-181.
- Dagogo-Jack, S., C. Rattarasarn and P. E. Cryer (1994). "Reversal of hypoglycemia unawareness, but not defective glucose counterregulation, in IDDM." Diabetes **43**(12): 1426-1434.
- Dagogo-Jack, S. E., S. Craft and P. E. Cryer (1993). "Hypoglycemia-associated autonomic failure in insulin-dependent diabetes mellitus. Recent antecedent hypoglycemia reduces autonomic responses to, symptoms of, and defense against subsequent hypoglycemia." The Journal of clinical investigation **91**(3): 819-828.
- Davis, M. R., M. Mellman and H. Shamoon (1992). "Further defects in counterregulatory responses induced by recurrent hypoglycemia in IDDM." Diabetes **41**(10): 1335-1340.
- Davis, M. R. and H. Shamoon (1991). "Counterregulatory adaptation to recurrent hypoglycemia in normal humans." The Journal of clinical endocrinology and metabolism **73**(5): 995-1001.
- Davis, S. N., S. Mann, P. Galassetti, R. A. Neill, D. Tate, A. C. Ertl and F. Costa (2000). "Effects of differing durations of antecedent hypoglycemia on counterregulatory responses to subsequent hypoglycemia in normal humans." Diabetes **49**(11): 1897-1903.
- Davis, S. N., C. Shavers, F. Costa and R. Mosqueda-Garcia (1996). "Role of cortisol in the pathogenesis of deficient counterregulation after antecedent hypoglycemia in normal humans." J Clin Invest **98**(3): 680-691.
- Davis, S. N., C. Shavers, F. Costa and R. Mosqueda-Garcia (1996). "Role of cortisol in the pathogenesis of deficient counterregulation after antecedent hypoglycemia in normal humans." The Journal of clinical investigation **98**(3): 680-691.
- De Feo, P., G. Perriello, E. Torlone, M. M. Ventura, C. Fanelli, F. Santeusano, P. Brunetti, J. E. Gerich and G. B. Bolli (1989). "Contribution of cortisol to glucose counterregulation in humans." The American journal of physiology **257**(1 Pt 1): E35-42.
- De Feyter, H. M., G. F. Mason, G. I. Shulman, D. L. Rothman and K. F. Petersen (2013). "Increased brain lactate concentrations without increased lactate oxidation during hypoglycemia in type 1 diabetic individuals." Diabetes **62**(9): 3075-3080.
- de Galan, B. E., C. J. Tack, J. W. Lenders, J. W. Pasman, L. D. Elving, F. G. Russel, J. A. Lutterman and P. Smits (2002). "Theophylline improves hypoglycemia unawareness in type 1 diabetes." Diabetes **51**(3): 790-796.
- de Kloet, E. R., M. Joels and F. Holsboer (2005). "Stress and the brain: from adaptation to disease." Nat Rev Neurosci **6**(6): 463-475.
- De Kloet, E. R., E. Vreugdenhil, M. S. Oitzl and M. Joels (1998). "Brain corticosteroid receptor balance in health and disease." Endocrine reviews **19**(3): 269-301.

de Vries, M. G., L. M. Arseneau, M. E. Lawson and J. L. Beverly (2003). "Extracellular glucose in rat ventromedial hypothalamus during acute and recurrent hypoglycemia." *Diabetes* **52**(11): 2767-2773.

de Zoysa, N., H. Rogers, M. Stadler, C. Gianfrancesco, S. Beveridge, E. Britneff, P. Choudhary, J. Elliott, S. Heller and S. A. Amiel (2014). "A psychoeducational program to restore hypoglycemia awareness: the DAFNE-HART pilot study." *Diabetes care* **37**(3): 863-866.

Deacon, C. F. (2011). "Dipeptidyl peptidase-4 inhibitors in the treatment of type 2 diabetes: a comparative review." *Diabetes, obesity & metabolism* **13**(1): 7-18.

Deary, I. J., D. A. Hepburn, K. M. MacLeod and B. M. Frier (1993). "Partitioning the symptoms of hypoglycaemia using multi-sample confirmatory factor analysis." *Diabetologia* **36**(8): 771-777.

Deary, I. J., D. Liewald and J. Nissan (2011). "A free, easy-to-use, computer-based simple and four-choice reaction time programme: the Deary-Liewald reaction time task." *Behav Res Methods* **43**(1): 258-268.

Debrah, K., R. S. Sherwin, J. Murphy and D. Kerr (1996). "Effect of caffeine on recognition of and physiological responses to hypoglycaemia in insulin-dependent diabetes." *Lancet* **347**(8993): 19-24.

DeFronzo, R. A., J. D. Tobin and R. Andres (1979). "Glucose clamp technique: a method for quantifying insulin secretion and resistance." *Am J Physiol* **237**(3): E214-223.

Deiss, D., J. Bolinder, J. P. Riveline, T. Battelino, E. Bosi, N. Tubiana-Rufi, D. Kerr and M. Phillip (2006). "Improved glycemic control in poorly controlled patients with type 1 diabetes using real-time continuous glucose monitoring." *Diabetes Care* **29**(12): 2730-2732.

Dekker, M. J., J. W. Koper, M. O. van Aken, H. A. Pols, A. Hofman, F. H. de Jong, C. Kirschbaum, J. C. Witteman, S. W. Lamberts and H. Tiemeier (2008). "Salivary cortisol is related to atherosclerosis of carotid arteries." *The Journal of clinical endocrinology and metabolism* **93**(10): 3741-3747.

DeRosa, M. A. and P. E. Cryer (2004). "Hypoglycemia and the sympathoadrenal system: neurogenic symptoms are largely the result of sympathetic neural, rather than adrenomedullary, activation." *American journal of physiology. Endocrinology and metabolism* **287**(1): E32-41.

Derr, R., E. Garrett, G. A. Stacy and C. D. Saudek (2003). "Is HbA(1c) affected by glycemic instability?" *Diabetes care* **26**(10): 2728-2733.

Deuschle, M., U. Gotthardt, U. Schweiger, B. Weber, A. Korner, J. Schmider, H. Standhardt, C. H. Lammers and I. Heuser (1997). "With aging in humans the activity of the hypothalamus-pituitary-adrenal system increases and its diurnal amplitude flattens." *Life sciences* **61**(22): 2239-2246.

DeVries, J. H., F. J. Snoek, P. J. Kostense, N. Masurel, R. J. Heine and G. Dutch Insulin Pump Study (2002). "A randomized trial of continuous subcutaneous insulin infusion and intensive injection therapy in type 1 diabetes for patients with long-standing poor glycemic control." *Diabetes Care* **25**(11): 2074-2080.

Diabetes, C., I. Complications Trial/Epidemiology of Diabetes, G. Complications Study Research, A. M. Jacobson, G. Musen, C. M. Ryan, N. Silvers, P. Cleary, B. Waberski, A. Burwood, K. Weinger, M. Bayless, W. Dahms and J. Harth (2007). "Long-term effect of diabetes and its treatment on cognitive function." *N Engl J Med* **356**(18): 1842-1852.

Direk, N., R. S. Newson, A. Hofman, C. Kirschbaum and H. Tiemeier (2011). "Short and long-term effects of smoking on cortisol in older adults." *International journal of psychophysiology : official journal of the International Organization of Psychophysiology* **80**(2): 157-160.

Dixon, W. G., M. Abrahamowicz, M. E. Beauchamp, D. W. Ray, S. Bernatsky, S. Suissa and M. P. Sylvestre (2012). "Immediate and delayed impact of oral glucocorticoid therapy on risk of serious infection in older patients with rheumatoid arthritis: a nested case-control analysis." *Annals of the rheumatic diseases* **71**(7): 1128-1133.

Dockray, S., M. R. Bhattacharyya, G. J. Molloy and A. Steptoe (2008). "The cortisol awakening response in relation to objective and subjective measures of waking in the morning." *Psychoneuroendocrinology* **33**(1): 77-82.

Donnelly, L. A., A. D. Morris, B. M. Frier, J. D. Ellis, P. T. Donnan, R. Durrant, M. M. Band, G. Reekie and G. P. Leese (2005). "Frequency and predictors of hypoglycaemia in Type 1 and insulin-treated

Type 2 diabetes: a population-based study." Diabetic medicine : a journal of the British Diabetic Association **22**(6): 749-755.

Donovan, C. M., P. Cane and R. N. Bergman (1991). "Search for the hypoglycemia receptor using the local irrigation approach." Advances in experimental medicine and biology **291**: 185-196.

Donovan, C. M. and A. G. Watts (2014). "Peripheral and central glucose sensing in hypoglycemic detection." Physiology (Bethesda) **29**(5): 314-324.

Drost, H., D. Gruneklee, H. K. Kley, W. Wiegelmann, H. L. Kruskemper and F. A. Gries (1980). "[Glucagon, growth hormone, and cortisol response to insulin-induced hypoglycemia in insulin-dependent diabetics (IDD) without autonomic neuropathy (author's transl)]." Klin Wochenschr **58**(21): 1197-1205.

Duckworth, W., C. Abraira, T. Moritz, D. Reda, N. Emanuele, P. D. Reaven, F. J. Zieve, J. Marks, S. N. Davis, R. Hayward, S. R. Warren, S. Goldman, M. McCarren, M. E. Vitek, W. G. Henderson and G. D. Huang (2009). "Glucose control and vascular complications in veterans with type 2 diabetes." The New England journal of medicine **360**(2): 129-139.

Duinkerken, E., A. M. Brands, E. van den Berg, J. M. Henselmans, R. P. Hoogma and G. J. Biessels (2011). "Cognition in older patients with type 1 diabetes mellitus: a longitudinal study." Journal of the American Geriatrics Society **59**(3): 563-565.

Dunlap, J. C. (1999). "Molecular bases for circadian clocks." Cell **96**(2): 271-290.

Dunn-Meynell, A. A., V. H. Routh, L. Kang, L. Gaspers and B. E. Levin (2002). "Glucokinase is the likely mediator of glucosensing in both glucose-excited and glucose-inhibited central neurons." Diabetes **51**(7): 2056-2065.

Dunn-Meynell, A. A., V. H. Routh, J. J. McArdle and B. E. Levin (1997). "Low-affinity sulfonylurea binding sites reside on neuronal cell bodies in the brain." Brain research **745**(1-2): 1-9.

Dunn, J. T., I. Cranston, P. K. Marsden, S. A. Amiel and L. J. Reed (2007). "Attenuation of amygdala and frontal cortical responses to low blood glucose concentration in asymptomatic hypoglycemia in type 1 diabetes: a new player in hypoglycemia unawareness?" Diabetes **56**(11): 2766-2773.

Dupre, J. (2005). "Glycaemic effects of incretins in Type 1 diabetes mellitus: a concise review, with emphasis on studies in humans." Regul Pept **128**(2): 149-157.

Dupre, J., M. T. Behme and T. J. McDonald (2004). "Exendin-4 normalized postcibal glycemic excursions in type 1 diabetes." The Journal of clinical endocrinology and metabolism **89**(7): 3469-3473.

Duran-Valdez, E., M. R. Burge, P. Broderick, L. Shey, V. Valentine, R. Schrader and D. S. Schade (2014). "Is the Masked Continuous Glucose Monitoring System Clinically Useful for Predicting Hemoglobin A1C in Type 1 Diabetes?" Diabetes technology & therapeutics **16**(5): 292-297.

Edgerton, D. S. and A. D. Cherrington (2011). "Glucagon as a critical factor in the pathology of diabetes." Diabetes **60**(2): 377-380.

Edwards, S., A. Clow, P. Evans and F. Hucklebridge (2001). "Exploration of the awakening cortisol response in relation to diurnal cortisol secretory activity." Life sciences **68**(18): 2093-2103.

Efrat, S., M. Tal and H. F. Lodish (1994). "The pancreatic beta-cell glucose sensor." Trends in biochemical sciences **19**(12): 535-538.

Egi, M. and R. Bellomo (2009). "Reducing glycemic variability in intensive care unit patients: a new therapeutic target?" Journal of diabetes science and technology **3**(6): 1302-1308.

El-Osta, A., D. Brasacchio, D. Yao, A. Poci, P. L. Jones, R. G. Roeder, M. E. Cooper and M. Brownlee (2008). "Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia." The Journal of experimental medicine **205**(10): 2409-2417.

Elliott, J., Heller, S. (2011). "Hypoglycaemia unawareness." Pract Diabetes Int **28**: 227-232.

Ellis, M. J., R. S. Schmidli, R. A. Donald, J. H. Livesey and E. A. Espiner (1990). "Plasma corticotrophin-releasing factor and vasopressin responses to hypoglycaemia in normal man." Clinical endocrinology **32**(1): 93-100.

Ellis, S. L., E. G. Moser, J. K. Snell-Bergeon, A. S. Rodionova, R. M. Hazenfield and S. K. Garg (2011). "Effect of sitagliptin on glucose control in adult patients with Type 1 diabetes: a pilot, double-blind,

randomized, crossover trial." Diabetic medicine : a journal of the British Diabetic Association **28**(10): 1176-1181.

Engvig, A., A. M. Fjell, L. T. Westlye, T. Moberget, O. Sundseth, V. A. Larsen and K. B. Walhovd (2012). "Memory training impacts short-term changes in aging white matter: a longitudinal diffusion tensor imaging study." Hum Brain Mapp **33**(10): 2390-2406.

Esposito, K., P. Chiodini, M. I. Maiorino, A. Capuano, D. Cozzolino, M. Petrizzo, G. Bellastella and D. Giugliano (2015). "A nomogram to estimate the HbA1c response to different DPP-4 inhibitors in type 2 diabetes: a systematic review and meta-analysis of 98 trials with 24 163 patients." BMJ Open **5**(2): e005892.

Evans, M. J., J. T. Brett, R. P. McIntosh, J. E. McIntosh, H. K. Roud, J. H. Livesey and R. A. Donald (1985). "The effect of various corticotropin-releasing factor trains on the release of adrenocorticotropin, beta-endorphin, and beta-lipotropin from perfused ovine pituitary cells." Endocrinology **117**(3): 893-899.

Evans, M. L., K. Matyka, J. Lomas, A. Pernet, I. C. Cranston, I. Macdonald and S. A. Amiel (1998). "Reduced counterregulation during hypoglycemia with raised circulating nonglucose lipid substrates: evidence for regional differences in metabolic capacity in the human brain?" The Journal of clinical endocrinology and metabolism **83**(8): 2952-2959.

Evans, M. L., R. J. McCrimmon, D. E. Flanagan, T. Keshavarz, X. Fan, E. C. McNay, R. J. Jacob and R. S. Sherwin (2004). "Hypothalamic ATP-sensitive K⁺ channels play a key role in sensing hypoglycemia and triggering counterregulatory epinephrine and glucagon responses." Diabetes **53**(10): 2542-2551.

Evans, M. L., A. Pernet, J. Lomas, J. Jones and S. A. Amiel (2000). "Delay in onset of awareness of acute hypoglycemia and of restoration of cognitive performance during recovery." Diabetes care **23**(7): 893-897.

Evans, P., F. Hucklebridge, C. Loveday and A. Clow (2012). "The cortisol awakening response is related to executive function in older age." International journal of psychophysiology : official journal of the International Organization of Psychophysiology **84**(2): 201-204.

Evans, P. D., C. Fredhøj, C. Loveday, F. Hucklebridge, E. Aitchison, D. Forte and A. Clow (2011). "The diurnal cortisol cycle and cognitive performance in the healthy old." International journal of psychophysiology : official journal of the International Organization of Psychophysiology **79**(3): 371-377.

Fadini, G. P. (2014). "A reappraisal of the role of circulating (progenitor) cells in the pathobiology of diabetic complications." Diabetologia **57**(1): 4-15.

Fadini, G. P., C. Agostini, S. Sartore and A. Avogaro (2007). "Endothelial progenitor cells in the natural history of atherosclerosis." Atherosclerosis **194**(1): 46-54.

Fadini, G. P., E. Boscaro, M. Albiero, L. Menegazzo, V. Frison, S. de Kreutzenberg, C. Agostini, A. Tiengo and A. Avogaro (2010). "The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1 α ." Diabetes care **33**(7): 1607-1609.

Fadini, G. P., E. Boscaro, S. de Kreutzenberg, C. Agostini, F. Seeger, S. Dimmeler, A. Zeiher, A. Tiengo and A. Avogaro (2010). "Time course and mechanisms of circulating progenitor cell reduction in the natural history of type 2 diabetes." Diabetes care **33**(5): 1097-1102.

Fadini, G. P., D. Losordo and S. Dimmeler (2012). "Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use." Circulation research **110**(4): 624-637.

Fan, X., Y. Ding, H. Cheng, D. X. Gram, R. S. Sherwin and R. J. McCrimmon (2008). "Amplified hormonal counterregulatory responses to hypoglycemia in rats after systemic delivery of a SUR-1-selective K⁺ channel opener?" Diabetes **57**(12): 3327-3334.

Fanelli, C., S. Pampanelli, L. Epifano, A. M. Rambotti, M. Ciofetta, F. Modarelli, A. Di Vincenzo, B. Annibale, M. Lepore, C. Lalli and et al. (1994). "Relative roles of insulin and hypoglycaemia on induction of neuroendocrine responses to, symptoms of, and deterioration of cognitive function in hypoglycaemia in male and female humans." Diabetologia **37**(8): 797-807.

Fanelli, C., S. Pampanelli, L. Epifano, A. M. Rambotti, A. Di Vincenzo, F. Modarelli, M. Ciofetta, M. Lepore, B. Annibale, E. Torlone and et al. (1994). "Long-term recovery from unawareness, deficient counterregulation and lack of cognitive dysfunction during hypoglycaemia, following institution of rational, intensive insulin therapy in IDDM." Diabetologia **37**(12): 1265-1276.

Fanelli, C., S. Pampanelli, C. Lalli, P. Del Sindaco, M. Ciofetta, M. Lepore, F. Porcellati, P. Bottini, A. Di Vincenzo, P. Brunetti and G. B. Bolli (1997). "Long-term intensive therapy of IDDM patients with clinically overt autonomic neuropathy: effects on hypoglycemia awareness and counterregulation." Diabetes **46**(7): 1172-1181.

Fanelli, C. G., C. S. Dence, J. Markham, T. O. Videen, D. S. Paramore, P. E. Cryer and W. J. Powers (1998). "Blood-to-brain glucose transport and cerebral glucose metabolism are not reduced in poorly controlled type 1 diabetes." Diabetes **47**(9): 1444-1450.

Fanelli, C. G., L. Epifano, A. M. Rambotti, S. Pampanelli, A. Di Vincenzo, F. Modarelli, M. Lepore, B. Annibale, M. Ciofetta and P. Bottini (1993). "Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM." Diabetes **42**(11): 1683-1689.

Fanelli, C. G., L. Epifano, A. M. Rambotti, S. Pampanelli, A. Di Vincenzo, F. Modarelli, M. Lepore, B. Annibale, M. Ciofetta, P. Bottini and et al. (1993). "Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM." Diabetes **42**(11): 1683-1689.

Fanelli, C. G., F. Porcellati, S. Pampanelli and G. B. Bolli (2004). "Insulin therapy and hypoglycaemia: the size of the problem." Diabetes/metabolism research and reviews **20 Suppl 2**: S32-42.

Farngren, J., M. Persson, A. Schweizer, J. E. Foley and B. Ahren (2012). "Vildagliptin reduces glucagon during hyperglycemia and sustains glucagon counterregulation during hypoglycemia in type 1 diabetes." The Journal of clinical endocrinology and metabolism **97**(10): 3799-3806.

Fellows, I. W., I. A. Macdonald, H. J. Wharrad and A. T. Birmingham (1986). "Low plasma concentrations of adrenaline and physiological tremor in man." J Neurol Neurosurg Psychiatry **49**(4): 396-399.

Fiallo-Scharer, R., J. Cheng, R. W. Beck, B. A. Buckingham, H. P. Chase, C. Kollman, L. Laffel, J. M. Lawrence, N. Mauras, W. V. Tamborlane, D. M. Wilson and H. Wolpert (2011). "Factors predictive of severe hypoglycemia in type 1 diabetes: analysis from the Juvenile Diabetes Research Foundation continuous glucose monitoring randomized control trial dataset." Diabetes care **34**(3): 586-590.

Finta, E. P., L. Harms, J. Sevcik, H. D. Fischer and P. Illes (1993). "Effects of potassium channel openers and their antagonists on rat locus coeruleus neurones." British journal of pharmacology **109**(2): 308-315.

Fioramonti, X., N. Marsollier, Z. Song, K. A. Fakira, R. M. Patel, S. Brown, T. Duparc, A. Pica-Mendez, N. M. Sanders, C. Knauf, P. Valet, R. J. McCrimmon, A. Beuve, C. Magnan and V. H. Routh (2010). "Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation." Diabetes **59**(2): 519-528.

Fisher, B. M., J. D. Quin, A. Rumley, S. E. Lennie, M. Small, A. C. MacCuish and G. D. Lowe (1991). "Effects of acute insulin-induced hypoglycaemia on haemostasis, fibrinolysis and haemorheology in insulin-dependent diabetic patients and control subjects." Clinical science **80**(5): 525-531.

Flanagan, D. E., T. Keshavarz, M. L. Evans, S. Flanagan, X. Fan, R. J. Jacob and R. S. Sherwin (2003). "Role of corticotrophin-releasing hormone in the impairment of counterregulatory responses to hypoglycemia." Diabetes **52**(3): 605-613.

Florez, J. C., N. Burt, P. I. de Bakker, P. Almgren, T. Tuomi, J. Holmkvist, D. Gaudet, T. J. Hudson, S. F. Schaffner, M. J. Daly, J. N. Hirschhorn, L. Groop and D. Altshuler (2004). "Haplotype structure and genotype-phenotype correlations of the sulfonylurea receptor and the islet ATP-sensitive potassium channel gene region." Diabetes **53**(5): 1360-1368.

Frier, B. (2009). "The incidence and impact of hypoglycemia in type 1 and type 2

diabetes." International Diabetes Monitor **21**: 210-218.

Frier, B., Fisher, BM (1999). Hypoglycaemia in Clinical Diabetes. Chichester, UK, John Wiley and Sons.

Frier, B. M. (2009). "Defining hypoglycaemia: what level has clinical relevance?" Diabetologia **52**(1): 31-34.

Frier, B. M., G. Schernthaner and S. R. Heller (2011). "Hypoglycemia and cardiovascular risks." Diabetes care **34 Suppl 2**: S132-137.

Fries, E., L. Dettenborn and C. Kirschbaum (2009). "The cortisol awakening response (CAR): facts and future directions." International journal of psychophysiology : official journal of the International Organization of Psychophysiology **72**(1): 67-73.

Fritsche, A., N. Stefan, H. Haring, J. Gerich and M. Stumvoll (2001). "Avoidance of hypoglycemia restores hypoglycemia awareness by increasing beta-adrenergic sensitivity in type 1 diabetes." Annals of internal medicine **134**(9 Pt 1): 729-736.

Fritsche, A., N. Stefan, H. Haring, J. Gerich and M. Stumvoll (2001). "Avoidance of hypoglycemia restores hypoglycemia awareness by increasing beta-adrenergic sensitivity in type 1 diabetes." Ann Intern Med **134**(9 Pt 1): 729-736.

Fruehwald-Schultes, B., W. Kern, W. Bong, P. Wellhoener, W. Kerner, J. Born, H. L. Fehm and A. Peters (1999). "Supraphysiological hyperinsulinemia acutely increases hypothalamic-pituitary-adrenal secretory activity in humans." The Journal of clinical endocrinology and metabolism **84**(9): 3041-3046.

Fukuda, M., A. Tanaka, Y. Tahara, H. Ikegami, Y. Yamamoto, Y. Kumahara and K. Shima (1988). "Correlation between minimal secretory capacity of pancreatic beta-cells and stability of diabetic control." Diabetes **37**(1): 81-88.

Gabriely, I. and H. Shamoon (2005). "Fructose normalizes specific counterregulatory responses to hypoglycemia in patients with type 1 diabetes." Diabetes **54**(3): 609-616.

Galassetti, P., S. Mann, D. Tate, R. A. Neill, F. Costa, D. H. Wasserman and S. N. Davis (2001). "Effects of antecedent prolonged exercise on subsequent counterregulatory responses to hypoglycemia." American journal of physiology. Endocrinology and metabolism **280**(6): E908-917.

Galloway, P. J., G. A. Thomson, B. M. Fisher and C. G. Semple (2000). "Insulin-induced hypoglycemia induces a rise in C-reactive protein." Diabetes care **23**(6): 861-862.

Garaulet, M. and J. A. Madrid (2009). "Chronobiology, genetics and metabolic syndrome." Current opinion in lipidology **20**(2): 127-134.

Garg, S., R. L. Brazg, T. S. Bailey, B. A. Buckingham, R. H. Slover, D. C. Klonoff, J. Shin, J. B. Welsh and F. R. Kaufman (2012). "Reduction in duration of hypoglycemia by automatic suspension of insulin delivery: the in-clinic ASPIRE study." Diabetes technology & therapeutics **14**(3): 205-209.

Garg, S., H. Zisser, S. Schwartz, T. Bailey, R. Kaplan, S. Ellis and L. Jovanovic (2006). "Improvement in glycemic excursions with a transcutaneous, real-time continuous glucose sensor: a randomized controlled trial." Diabetes care **29**(1): 44-50.

Garg, S. K., R. L. Brazg, T. S. Bailey, B. A. Buckingham, R. H. Slover, D. C. Klonoff, J. Shin, J. B. Welsh and F. R. Kaufman (2014). "Hypoglycemia Begets Hypoglycemia: The Order Effect in the ASPIRE In-Clinic Study." Diabetes technology & therapeutics **16**(3): 125-130.

Garg, S. K., E. G. Moser, B. W. Bode, L. J. Klaff, W. R. Hiatt, C. Beatson and J. K. Snell-Bergeon (2013). "Effect of sitagliptin on post-prandial glucagon and GLP-1 levels in patients with type 1 diabetes: investigator-initiated, double-blind, randomized, placebo-controlled trial." Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists **19**(1): 19-28.

Gaudino, E. A., M. W. Geisler and N. K. Squires (1995). "Construct validity in the Trail Making Test: what makes Part B harder?" Journal of clinical and experimental neuropsychology **17**(4): 529-535.

Geddes, J., J. E. Schopman, N. N. Zammitt and B. M. Frier (2008). "Prevalence of impaired awareness of hypoglycaemia in adults with Type 1 diabetes." Diabet Med **25**(4): 501-504.

Geddes, J., R. J. Wright, N. N. Zammitt, I. J. Deary and B. M. Frier (2007). "An evaluation of methods of assessing impaired awareness of hypoglycemia in type 1 diabetes." Diabetes Care **30**(7): 1868-1870.

Gedulin, B. R., S. E. Nikoulina, P. A. Smith, G. Gedulin, L. L. Nielsen, A. D. Baron, D. G. Parkes and A. A. Young (2005). "Exenatide (exendin-4) improves insulin sensitivity and β -cell mass in insulin-resistant obese fa/fa Zucker rats independent of glycemia and body weight." Endocrinology **146**(4): 2069-2076.

Geho, W. B. (2014). "The importance of the liver in insulin replacement therapy in insulin-deficient diabetes." Diabetes **63**(5): 1445-1447.

Geiss, A., E. Varadi, K. Steinbach, H. W. Bauer and F. Anton (1997). "Psychoneuroimmunological correlates of persisting sciatic pain in patients who underwent discectomy." Neuroscience letters **237**(2-3): 65-68.

George, E., N. Harris, C. Bedford, I. A. Macdonald, C. A. Hardisty and S. R. Heller (1995). "Prolonged but partial impairment of the hypoglycaemic physiological response following short-term hypoglycaemia in normal subjects." Diabetologia **38**(10): 1183-1190.

Gerich, J. E., M. Langlois, C. Noacco, J. H. Karam and P. H. Forsham (1973). "Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect." Science **182**(4108): 171-173.

Gerich, J. E., M. Langlois, C. Noacco, J. H. Karam and P. H. Forsham (1973). "Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect." Science **182**(4108): 171-173.

Gerich, J. E., M. Lorenzi, D. M. Bier, V. Schneider, E. Tsalikian, J. H. Karam and P. H. Forsham (1975). "Prevention of human diabetic ketoacidosis by somatostatin. Evidence for an essential role of glucagon." N Engl J Med **292**(19): 985-989.

Gerin, W. and T. G. Pickering (1995). "Association between delayed recovery of blood pressure after acute mental stress and parental history of hypertension." Journal of hypertension **13**(6): 603-610.

Gerstein, H. C., M. E. Miller, R. P. Byington, D. C. Goff, Jr., J. T. Bigger, J. B. Buse, W. C.ushman, S. Genuth, F. Ismail-Beigi, R. H. Grimm, Jr., J. L. Probstfield, D. G. Simons-Morton and W. T. Friedewald (2008). "Effects of intensive glucose lowering in type 2 diabetes." The New England journal of medicine **358**(24): 2545-2559.

Ghizzoni, L., M. Vanelli, R. Virdis, A. Alberini, C. Volta and S. Bernasconi (1993). "Adrenal steroid and adrenocorticotropin responses to human corticotropin-releasing hormone stimulation test in adolescents with type I diabetes mellitus." Metabolism: clinical and experimental **42**(9): 1141-1145.

Giacco, F. and M. Brownlee (2010). "Oxidative stress and diabetic complications." Circulation research **107**(9): 1058-1070.

Giampietro, O., C. Giampietro, L. D. Bartola, M. C. Masoni and E. Matteucci (2013). "Sitagliptin as add-on therapy in insulin deficiency: biomarkers of therapeutic efficacy respond differently in type 1 and type 2 diabetes." Drug design, development and therapy **7**: 99-104.

Gilon, P., Rui Cheng-Xue, Ana Gómez-Ruiz, Antoine, N., Hee-young Chae (2014). "The control of glucagon secretion by glucose and KATP channel modulators." Proceedings of the Belgian Royal College of Academies of Medicine **3**: 16.

Gimenez, M., R. Gilabert, J. Monteagudo, A. Alonso, R. Casamitjana, C. Pare and I. Conget (2011). "Repeated episodes of hypoglycemia as a potential aggravating factor for preclinical atherosclerosis in subjects with type 1 diabetes." Diabetes Care **34**(1): 198-203.

Gimenez, M., M. Lara and I. Conget (2010). "Sustained efficacy of continuous subcutaneous insulin infusion in type 1 diabetes subjects with recurrent non-severe and severe hypoglycemia and hypoglycemia unawareness: a pilot study." Diabetes technology & therapeutics **12**(7): 517-521.

Gimenez, M., J. J. Lopez, C. Castell and I. Conget (2012). "Hypoglycaemia and cardiovascular disease in Type 1 Diabetes. Results from the Catalan National Public Health registry on insulin pump therapy." Diabetes research and clinical practice **96**(2): e23-25.

Gloyn, A. L., Y. Hashim, S. J. Ashcroft, R. Ashfield, S. Wiltshire, R. C. Turner and U. K. P. D. Study (2001). "Association studies of variants in promoter and coding regions of beta-cell ATP-sensitive K-channel genes SUR1 and Kir6.2 with Type 2 diabetes mellitus (UKPDS 53)." Diabet Med **18**(3): 206-212.

Gogitidze Joy, N., M. S. Hedrington, V. J. Briscoe, D. B. Tate, A. C. Ertl and S. N. Davis (2010). "Effects of acute hypoglycemia on inflammatory and pro-atherothrombotic biomarkers in individuals with type 1 diabetes and healthy individuals." Diabetes care **33**(7): 1529-1535.

Gold, A. E., I. J. Deary and B. M. Frier (1997). "Hypoglycaemia and non-cognitive aspects of psychological function in insulin-dependent (type 1) diabetes mellitus (IDDM)." Diabet Med **14**(2): 111-118.

Gold, A. E., K. M. MacLeod and B. M. Frier (1994). "Frequency of severe hypoglycemia in patients with type I diabetes with impaired awareness of hypoglycemia." Diabetes care **17**(7): 697-703.

Gold, P. W., H. Gwirtsman, P. C. Avgerinos, L. K. Nieman, W. T. Gallucci, W. Kaye, D. Jimerson, M. Ebert, R. Rittmaster, D. L. Loriaux and et al. (1986). "Abnormal hypothalamic-pituitary-adrenal function in anorexia nervosa. Pathophysiologic mechanisms in underweight and weight-corrected patients." The New England journal of medicine **314**(21): 1335-1342.

Gold, P. W., D. L. Loriaux, A. Roy, M. A. Kling, J. R. Calabrese, C. H. Kellner, L. K. Nieman, R. M. Post, D. Pickar, W. Gallucci and et al. (1986). "Responses to corticotropin-releasing hormone in the hypercortisolism of depression and Cushing's disease. Pathophysiologic and diagnostic implications." The New England journal of medicine **314**(21): 1329-1335.

Gordon, M. K., E. Peloso, A. Auken and M. Dozier (2005). "Effect of flavored beverage crystals on salivary cortisol enzyme-immunoreactive assay measurements." Developmental psychobiology **47**(2): 189-195.

Graeff, F. G., C. Garcia-Leal, C. M. Del-Ben and F. S. Guimaraes (2005). "Does the panic attack activate the hypothalamic-pituitary-adrenal axis?" An Acad Bras Cienc **77**(3): 477-491.

Gragoli, C. (2014). "Hypothesis of the neuroendocrine cortisol pathway gene role in the comorbidity of depression, type 2 diabetes, and metabolic syndrome." Appl Clin Genet **7**: 43-53.

Graveling, A. J., I. J. Deary and B. M. Frier (2013). "Acute hypoglycemia impairs executive cognitive function in adults with and without type 1 diabetes." Diabetes care **36**(10): 3240-3246.

Graveling, A. J. and B. M. Frier (2010). "Impaired awareness of hypoglycaemia: a review." Diabetes Metab **36 Suppl 3**: S64-74.

Graveling, A. J., K. J. Noyes, M. H. Allerhand, R. J. Wright, L. E. Bath, I. J. Deary and B. M. Frier (2014). "Prevalence of impaired awareness of hypoglycemia and identification of predictive symptoms in children and adolescents with type 1 diabetes." Pediatr Diabetes **15**(3): 206-213.

Gromada, J., I. Franklin and C. B. Wollheim (2007). "Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains." Endocrine reviews **28**(1): 84-116.

Grossi, G., A. Perski, U. Lundberg and J. Soares (2001). "Associations between financial strain and the diurnal salivary cortisol secretion of long-term unemployed individuals." Integrative physiological and behavioral science : the official journal of the Pavlovian Society **36**(3): 205-219.

group, D. (1991). "Epidemiology of severe hypoglycemia in the diabetes control and complications trial. The DCCT Research Group." Am J Med **90**(4): 450-459.

group, D. (1993). "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group." N Engl J Med **329**(14): 977-986.

group, D. (1997). "Hypoglycemia in the Diabetes Control and Complications Trial. The Diabetes Control and Complications Trial Research Group." Diabetes **46**(2): 271-286.

Group, U. K. H. S. (2007). "Risk of hypoglycaemia in types 1 and 2 diabetes: effects of treatment modalities and their duration." Diabetologia **50**(6): 1140-1147.

Gulanski, B. I., H. M. De Feyter, K. A. Page, R. B. de Aguiar, G. F. Mason, D. L. Rothman and R. S. Sherwin (2013). "Increased Brain Transport and Metabolism of Acetate in Hypoglycemia Unawareness." The Journal of clinical endocrinology and metabolism.

Gustavson, S. M., C. A. Chu, M. Nishizawa, B. Farmer, D. Neal, Y. Yang, E. P. Donahue, P. Flakoll and A. D. Cherrington (2003). "Interaction of glucagon and epinephrine in the control of hepatic glucose production in the conscious dog." American journal of physiology. Endocrinology and metabolism **284**(4): E695-707.

Hadi, H. A. and J. A. Suwaidi (2007). "Endothelial dysfunction in diabetes mellitus." Vascular health and risk management **3**(6): 853-876.

Halcox, J. P., A. E. Donald, E. Ellins, D. R. Witte, M. J. Shipley, E. J. Brunner, M. G. Marmot and J. E. Deanfield (2009). "Endothelial function predicts progression of carotid intima-media thickness." Circulation **119**(7): 1005-1012.

Hamming, K. S., D. Soliman, L. C. Maternisz, O. Niazi, Y. Lang, A. L. Gloyn and P. E. Light (2009). "Coexpression of the type 2 diabetes susceptibility gene variants KCNJ11 E23K and ABCC8 S1369A alter the ATP and sulfonylurea sensitivities of the ATP-sensitive K(+) channel." Diabetes **58**(10): 2419-2424.

Hammond, G. L. and M. S. Langley (1986). "Identification and measurement of sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG) in human saliva." Acta endocrinologica **112**(4): 603-608.

Han, S. M., C. Namkoong, P. G. Jang, I. S. Park, S. W. Hong, H. Katakami, S. Chun, S. W. Kim, J. Y. Park, K. U. Lee and M. S. Kim (2005). "Hypothalamic AMP-activated protein kinase mediates counter-regulatory responses to hypoglycaemia in rats." Diabetologia **48**(10): 2170-2178.

Hani, E. H., P. Boutin, E. Durand, H. Inoue, M. A. Permutt, G. Velho and P. Froguel (1998). "Missense mutations in the pancreatic islet beta cell inwardly rectifying K+ channel gene (KIR6.2/BIR): a meta-analysis suggests a role in the polygenic basis of Type II diabetes mellitus in Caucasians." Diabetologia **41**(12): 1511-1515.

Hardy, K. J., M. R. Burge, P. J. Boyle and J. H. Scarpello (1994). "A treatable cause of recurrent severe hypoglycemia." Diabetes care **17**(7): 722-724.

Hari Kumar, K. V., A. Shaikh and P. Prusty (2013). "Addition of exenatide or sitagliptin to insulin in new onset type 1 diabetes: a randomized, open label study." Diabetes research and clinical practice **100**(2): e55-58.

Hauger, R. L. and G. Aguilera (1993). "Regulation of pituitary corticotropin releasing hormone (CRH) receptors by CRH: interaction with vasopressin." Endocrinology **133**(4): 1708-1714.

Heim, C., U. Ehlerth and D. H. Hellhammer (2000). "The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders." Psychoneuroendocrinology **25**(1): 1-35.

Heller, S. R. and P. E. Cryer (1991). "Reduced neuroendocrine and symptomatic responses to subsequent hypoglycemia after 1 episode of hypoglycemia in nondiabetic humans." Diabetes **40**(2): 223-226.

Heller, S. R. and I. A. Macdonald (1996). "The measurement of cognitive function during acute hypoglycaemia: experimental limitations and their effect on the study of hypoglycaemia unawareness." Diabet Med **13**(7): 607-615.

Hellhammer, J., E. Fries, O. W. Schweithal, W. Schlotz, A. A. Stone and D. Hagemann (2007). "Several daily measurements are necessary to reliably assess the cortisol rise after awakening: state- and trait components." Psychoneuroendocrinology **32**(1): 80-86.

Hendrickson, A. E., N. Wagoner and W. M. Cowan (1972). "An autoradiographic and electron microscopic study of retino-hypothalamic connections." Zeitschrift fur Zellforschung und mikroskopische Anatomie **135**(1): 1-26.

Henley, D. E., J. A. Leendertz, G. M. Russell, S. A. Wood, S. Taheri, W. W. Woltersdorf and S. L. Lightman (2009). "Development of an automated blood sampling system for use in humans." J Med Eng Technol **33**(3): 199-208.

Henry, R. R., J. Rosenstock, S. Edelman, S. Mudaliar, A. G. Chalamandaris, S. Kasichayanula, A. Bogle, N. Iqbal, J. List and S. C. Griffen (2015). "Exploring the potential of the SGLT2 inhibitor dapagliflozin in type 1 diabetes: a randomized, double-blind, placebo-controlled pilot study." Diabetes care **38**(3): 412-419.

Henry, R. R., S. R. Smith, S. L. Schwartz, S. R. Mudaliar, C. F. Deacon, J. J. Holst, R. Y. Duan, R. S. Chen and J. F. List (2011). "Effects of saxagliptin on beta-cell stimulation and insulin secretion in patients with type 2 diabetes." Diabetes Obes Metab **13**(9): 850-858.

Hepburn, D., Ed. (1993). Symptoms of hypoglycaemia. In Hypoglycaemia and Diabetes; Clinical and Physiological Aspects. London, Edward Arnold.

Heptulla, R. A., W. V. Tamborlane, T. Y. Ma, F. Rife and R. S. Sherwin (2001). "Oral glucose augments the counterregulatory hormone response during insulin-induced hypoglycemia in humans." The Journal of clinical endocrinology and metabolism **86**(2): 645-648.

Hermanides, J., T. M. Vriesendorp, R. J. Bosman, D. F. Zandstra, J. B. Hoekstra and J. H. Devries (2010). "Glucose variability is associated with intensive care unit mortality." Critical care medicine **38**(3): 838-842.

Herzog, E. D. (2007). "Neurons and networks in daily rhythms." Nat Rev Neurosci **8**(10): 790-802.

Herzog, R. I., O. Chan, S. Yu, J. Dziura, E. C. McNay and R. S. Sherwin (2008). "Effect of acute and recurrent hypoglycemia on changes in brain glycogen concentration." Endocrinology **149**(4): 1499-1504.

Hevener, A. L., R. N. Bergman and C. M. Donovan (1997). "Novel glucosensor for hypoglycemic detection localized to the portal vein." Diabetes **46**(9): 1521-1525.

Hill, N. R., N. S. Oliver, P. Choudhary, J. C. Levy, P. Hindmarsh and D. R. Matthews (2011). "Normal reference range for mean tissue glucose and glycemic variability derived from continuous glucose monitoring for subjects without diabetes in different ethnic groups." Diabetes technology & therapeutics **13**(9): 921-928.

Hilsted, J. (1993). "Cardiovascular changes during hypoglycaemia." Clinical physiology **13**(1): 1-10.

Hoffman, R. P., S. Arslanian, A. L. Drash and D. J. Becker (1994). "Impaired counterregulatory hormone responses to hypoglycemia in children and adolescents with new onset IDDM." J Pediatr Endocrinol **7**(3): 235-244.

Hoheisel, G., J. Schauer, W. A. Scherbaum and S. R. Bornstein (1998). "The effect of corticotropin-releasing hormone (CRH) on the adrenal medulla in hypophysectomized rats." Histol Histopathol **13**(1): 81-87.

Honda, K., H. Kamisoyama, T. Uemura, T. Yanagi, N. Saito, Y. Kurose, K. Sugahara, K. Katoh and S. Hasegawa (2012). "The mechanism underlying the central glucagon-induced hyperglycemia and anorexia in chicks." Comp Biochem Physiol A Mol Integr Physiol **163**(3-4): 260-264.

Hopkins, D., I. Lawrence, P. Mansell, G. Thompson, S. Amiel, M. Campbell and S. Heller (2012). "Improved biomedical and psychological outcomes 1 year after structured education in flexible insulin therapy for people with type 1 diabetes: the U.K. DAFNE experience." Diabetes Care **35**(8): 1638-1642.

Howell, S. L. (1984). "The mechanism of insulin secretion." Diabetologia **26**(5): 319-327.

Hucklebridge, F., T. Hussain, P. Evans and A. Clow (2005). "The diurnal patterns of the adrenal steroids cortisol and dehydroepiandrosterone (DHEA) in relation to awakening." Psychoneuroendocrinology **30**(1): 51-57.

Hudson, J. I., M. S. Hudson, A. J. Rothschild, L. Vignati, A. F. Schatzberg and J. C. Melby (1984). "Abnormal results of dexamethasone suppression tests in nondepressed patients with diabetes mellitus." Archives of general psychiatry **41**(11): 1086-1089.

Hutton, R. A., D. Mikhailidis, K. M. Dormandy and J. Ginsburg (1979). "Platelet aggregation studies during transient hypoglycaemia: a potential method for evaluating platelet function." J Clin Pathol **32**(5): 434-438.

Ide, T., H. Tsutsui, N. Ohashi, S. Hayashidani, N. Suematsu, M. Tsuchihashi, H. Tamai and A. Takeshita (2002). "Greater oxidative stress in healthy young men compared with premenopausal women." Arteriosclerosis, thrombosis, and vascular biology **22**(3): 438-442.

Ionut, V., A. V. Castro, O. O. Woolcott, D. Stefanovski, M. S. Iyer, J. L. Broussard, H. Mkrtchyan, M. Burch, R. Elazary, E. Kirkman and R. N. Bergman (2014). "Essentiality of portal vein receptors in

hypoglycemic counterregulation: direct proof via denervation in male canines." Endocrinology **155**(4): 1247-1254.

Isler, M., N. Delibas, M. Guclu, F. Gultekin, R. Sutcu, M. Bahceci and A. Kosar (2002). "Superoxide dismutase and glutathione peroxidase in erythrocytes of patients with iron deficiency anemia: effects of different treatment modalities." Croatian medical journal **43**(1): 16-19.

Jacobson, L., T. Ansari and O. P. McGuinness (2006). "Counterregulatory deficits occur within 24 h of a single hypoglycemic episode in conscious, unrestrained, chronically cannulated mice." American journal of physiology. Endocrinology and metabolism **290**(4): E678-684.

Jaffrey, S. R., H. Erdjument-Bromage, C. D. Ferris, P. Tempst and S. H. Snyder (2001). "Protein S-nitrosylation: a physiological signal for neuronal nitric oxide." Nat Cell Biol **3**(2): 193-197.

Jahanshahi, M., M. Torkamani, M. Beigi, L. Wilkinson, D. Page, L. Madeley, K. Bhatia, M. Hariz, L. Zrinzo, P. Limousin, D. Ruge and S. Tisch (2014). "Pallidal stimulation for primary generalised dystonia: effect on cognition, mood and quality of life." J Neurol **261**(1): 164-173.

Jaiswal, M., K. McKeon, N. Comment, J. Henderson, S. Swanson, C. Plunkett, P. Nelson and R. Pop-Busui (2014). "Association Between Impaired Cardiovascular Autonomic Function and Hypoglycemia in Patients With Type 1 Diabetes." Diabetes care.

James, F. O., C. D. Walker and D. B. Boivin (2004). "Controlled exposure to light and darkness realigns the salivary cortisol rhythm in night shift workers." Chronobiology international **21**(6): 961-972.

Janssen, M. M., F. J. Snoek, R. T. de Jongh, S. Casteleijn, W. Deville and R. J. Heine (2000). "Biological and behavioural determinants of the frequency of mild, biochemical hypoglycaemia in patients with Type 1 diabetes on multiple insulin injection therapy." Diabetes/metabolism research and reviews **16**(3): 157-163.

Janssen, M. M., F. J. Snoek and R. J. Heine (2000). "Assessing impaired hypoglycemia awareness in type 1 diabetes: agreement of self-report but not of field study data with the autonomic symptom threshold during experimental hypoglycemia." Diabetes care **23**(4): 529-532.

Jasper, M. S. and W. C. Engeland (1997). "Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats." The American journal of physiology **273**(2 Pt 1): E363-368.

Javorsky, M., L. Klimcakova, Z. Schroner, J. Zidzik, E. Babjakova, M. Fabianova, M. Kozarova, R. Tkacova, J. Salagovic and I. Tkac (2012). "KCNJ11 gene E23K variant and therapeutic response to sulfonylureas." Eur J Intern Med **23**(3): 245-249.

Jitrapakdee, S. (2012). "Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis." The international journal of biochemistry & cell biology **44**(1): 33-45.

Jones, S. C., H. J. Saunders, W. Qi and C. A. Pollock (1999). "Intermittent high glucose enhances cell growth and collagen synthesis in cultured human tubulointerstitial cells." Diabetologia **42**(9): 1113-1119.

Juonala, M., J. S. Viikari, T. Laitinen, J. Marniemi, H. Helenius, T. Ronnema and O. T. Raitakari (2004). "Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: the cardiovascular risk in young Finns study." Circulation **110**(18): 2918-2923.

Juvenile Diabetes Research Foundation Continuous Glucose Monitoring Study, G., W. V. Tamborlane, R. W. Beck, B. W. Bode, B. Buckingham, H. P. Chase, R. Clemons, R. Fiallo-Scharer, L. A. Fox, L. K. Gilliam, I. B. Hirsch, E. S. Huang, C. Kollman, A. J. Kowalski, L. Laffel, J. M. Lawrence, J. Lee, N. Maura, M. O'Grady, K. J. Ruedy, M. Tansey, E. Tsalikian, S. Weinzimer, D. M. Wilson, H. Wolpert, T. Wysocki and D. Xing (2008). "Continuous glucose monitoring and intensive treatment of type 1 diabetes." N Engl J Med **359**(14): 1464-1476.

Kamath, A., A. Mahalingam and J. Brauker (2010). "Methods of evaluating the utility of continuous glucose monitor alerts." J Diabetes Sci Technol **4**(1): 57-66.

Kamp, F., N. Kizilbash, B. E. Corkey, P. O. Berggren and J. A. Hamilton (2003). "Sulfonylureas rapidly cross phospholipid bilayer membranes by a free-diffusion mechanism." Diabetes **52**(10): 2526-2531.

Kang, L., A. A. Dunn-Meynell, V. H. Routh, L. D. Gaspers, Y. Nagata, T. Nishimura, J. Eiki, B. B. Zhang and B. E. Levin (2006). "Glucokinase is a critical regulator of ventromedial hypothalamic neuronal glucosensing." Diabetes **55**(2): 412-420.

Kang, L., V. H. Routh, E. V. Kuzhikandathil, L. D. Gaspers and B. E. Levin (2004). "Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons." Diabetes **53**(3): 549-559.

Katsuno, T., H. Ikeda, K. Ida, J. Miyagawa and M. Namba (2013). "Add-on therapy with the DPP-4 inhibitor sitagliptin improves glycemic control in insulin-treated Japanese patients with type 2 diabetes mellitus." Endocrine journal **60**(6): 733-742.

Keenan, D. B., J. J. Mastrototaro, H. Zisser, K. A. Cooper, G. Raghavendhar, S. W. Lee, J. Yusi, T. S. Bailey, R. L. Brazg and R. V. Shah (2012). "Accuracy of the Enlite 6-day glucose sensor with guardian and Veo calibration algorithms." Diabetes technology & therapeutics **14**(3): 225-231.

Kielgast, U., M. Asmar, S. Madsbad and J. J. Holst (2010). "Effect of glucagon-like peptide-1 on alpha- and beta-cell function in C-peptide-negative type 1 diabetic patients." The Journal of clinical endocrinology and metabolism **95**(5): 2492-2496.

Kielgast, U., J. J. Holst and S. Madsbad (2011). "Antidiabetic actions of endogenous and exogenous GLP-1 in type 1 diabetic patients with and without residual beta-cell function." Diabetes **60**(5): 1599-1607.

Kilpatrick, E. S., A. S. Rigby and S. L. Atkin (2008). "A1C variability and the risk of microvascular complications in type 1 diabetes: data from the Diabetes Control and Complications Trial." Diabetes care **31**(11): 2198-2202.

Kilpatrick, E. S., A. S. Rigby and S. L. Atkin (2008). "Mean blood glucose compared with HbA1c in the prediction of cardiovascular disease in patients with type 1 diabetes." Diabetologia **51**(2): 365-371.

Kilpatrick, E. S., A. S. Rigby and S. L. Atkin (2009). "Effect of glucose variability on the long-term risk of microvascular complications in type 1 diabetes." Diabetes care **32**(10): 1901-1903.

Kilpatrick, E. S., A. S. Rigby, K. Goode and S. L. Atkin (2007). "Relating mean blood glucose and glucose variability to the risk of multiple episodes of hypoglycaemia in type 1 diabetes." Diabetologia **50**(12): 2553-2561.

King, P., M. F. Kong, H. Parkin, I. A. Macdonald and R. B. Tattersall (1998). "Well-being, cerebral function, and physical fatigue after nocturnal hypoglycemia in IDDM." Diabetes Care **21**(3): 341-345.

Kirschbaum, C. and D. H. Hellhammer (1994). "Salivary cortisol in psychoneuroendocrine research: recent developments and applications." Psychoneuroendocrinology **19**(4): 313-333.

Kirschbaum, C. and D. H. Hellhammer (1999). "Noise and Stress - Salivary Cortisol as a Non-Invasive Measure of Allostatic Load." Noise Health **1**(4): 57-66.

Kirschbaum, C., J. C. Prussner, A. A. Stone, I. Federenko, J. Gaab, D. Lintz, N. Schommer and D. H. Hellhammer (1995). "Persistent high cortisol responses to repeated psychological stress in a subpopulation of healthy men." Psychosomatic medicine **57**(5): 468-474.

Kirschbaum, C., O. T. Wolf, M. May, W. Wippich and D. H. Hellhammer (1996). "Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults." Life sciences **58**(17): 1475-1483.

Kishore, P., L. Boucai, K. Zhang, W. Li, S. Koppaka, S. Kehlenbrink, A. Schiwek, Y. B. Esterson, D. Mehta, S. Bursheh, Y. Su, R. Gutierrez-Juarez, R. Muzumdar, G. J. Schwartz and M. Hawkins (2011). "Activation of K(ATP) channels suppresses glucose production in humans." The Journal of clinical investigation **121**(12): 4916-4920.

Klein, R. (1995). "Hyperglycemia and microvascular and macrovascular disease in diabetes." Diabetes Care **18**(2): 258-268.

Kohnert, K. D., P. Heinke, G. Fritzsche, L. Vogt, P. Augstein and E. Salzsieder (2013). "Evaluation of the mean absolute glucose change as a measure of glycemic variability using continuous glucose monitoring data." Diabetes technology & therapeutics **15**(6): 448-454.

Kolanowski, J., M. A. Pizarro and Crabbe (1975). "Potentiation of adrenocortical response upon intermittent stimulation with corticotropin in normal subjects." The Journal of clinical endocrinology and metabolism **41**(3): 453-465.

Koranyi, L., R. E. Bourey, D. James, M. Mueckler, F. T. Fiedorek, Jr. and M. A. Permutt (1991). "Glucose transporter gene expression in rat brain: Pretranslational changes associated with chronic insulin-induced hypoglycemia, fasting, and diabetes." Mol Cell Neurosci **2**(3): 244-252.

Kortte, K. B., M. D. Horner and W. K. Windham (2002). "The trail making test, part B: cognitive flexibility or ability to maintain set?" Appl Neuropsychol **9**(2): 106-109.

Kovatchev, B. P., D. J. Cox, L. S. Farhy, M. Straume, L. Gonder-Frederick and W. L. Clarke (2000). "Episodes of severe hypoglycemia in type 1 diabetes are preceded and followed within 48 hours by measurable disturbances in blood glucose." J Clin Endocrinol Metab **85**(11): 4287-4292.

Kovatchev, B. P., D. J. Cox, L. S. Farhy, M. Straume, L. Gonder-Frederick and W. L. Clarke (2000). "Episodes of severe hypoglycemia in type 1 diabetes are preceded and followed within 48 hours by measurable disturbances in blood glucose." The Journal of clinical endocrinology and metabolism **85**(11): 4287-4292.

Kovatchev, B. P., D. J. Cox, L. A. Gonder-Frederick and W. Clarke (1997). "Symmetrization of the blood glucose measurement scale and its applications." Diabetes care **20**(11): 1655-1658.

Kovatchev, B. P., D. J. Cox, L. A. Gonder-Frederick, D. Young-Hyman, D. Schlundt and W. Clarke (1998). "Assessment of risk for severe hypoglycemia among adults with IDDM: validation of the low blood glucose index." Diabetes care **21**(11): 1870-1875.

Kovatchev, B. P., D. J. Cox, A. Kumar, L. Gonder-Frederick and W. L. Clarke (2003). "Algorithmic evaluation of metabolic control and risk of severe hypoglycemia in type 1 and type 2 diabetes using self-monitoring blood glucose data." Diabetes technology & therapeutics **5**(5): 817-828.

Kovatchev, B. P., E. Otto, D. Cox, L. Gonder-Frederick and W. Clarke (2006). "Evaluation of a new measure of blood glucose variability in diabetes." Diabetes care **29**(11): 2433-2438.

Kramer, C. K., C. A. Borgono, P. Van Nostrand, R. Retnakaran and B. Zinman (2014). "Glucagon response to oral glucose challenge in type 1 diabetes: lack of impact of euglycemia." Diabetes care **37**(4): 1076-1082.

Krieger, D. T., W. Allen, F. Rizzo and H. P. Krieger (1971). "Characterization of the normal temporal pattern of plasma corticosteroid levels." The Journal of clinical endocrinology and metabolism **32**(2): 266-284.

Kubiak, T., N. Hermanns, H. J. Schreckling, B. Kulzer and T. Haak (2004). "Assessment of hypoglycaemia awareness using continuous glucose monitoring." Diabetic medicine : a journal of the British Diabetic Association **21**(5): 487-490.

Kudielka, B. M., S. Bellingrath and D. H. Hellhammer (2006). "Cortisol in burnout and vital exhaustion: an overview." Giornale italiano di medicina del lavoro ed ergonomia **28**(1 Suppl 1): 34-42.

Kudielka, B. M., J. E. Broderick and C. Kirschbaum (2003). "Compliance with saliva sampling protocols: electronic monitoring reveals invalid cortisol daytime profiles in noncompliant subjects." Psychosomatic medicine **65**(2): 313-319.

Kuenen, J. C., R. Borg, D. J. Kuik, H. Zheng, D. Schoenfeld, M. Diamant, D. M. Nathan and R. J. Heine (2011). "Does glucose variability influence the relationship between mean plasma glucose and HbA1c levels in type 1 and type 2 diabetic patients?" Diabetes care **34**(8): 1843-1847.

Kuhadiya, N. (2014). Liraglutide plus insulin improved HbA1c, body weight, QOL in patients with Type 1 Diabetes. AACE 23rd Annual Scientific & Clinical Congress May 13-18, Las Vegas, Nevada.

Kuhara, T., S. Ikeda, A. Ohneda and Y. Sasaki (1991). "Effects of intravenous infusion of 17 amino acids on the secretion of GH, glucagon, and insulin in sheep." The American journal of physiology **260**(1 Pt 1): E21-26.

Kumari, M., E. Badrick, T. Chandola, N. E. Adler, E. Epel, T. Seeman, C. Kirschbaum and M. G. Marmot (2010). "Measures of social position and cortisol secretion in an aging population: findings from the Whitehall II study." Psychosomatic medicine **72**(1): 27-34.

Kumari, M., E. Badrick, A. Sacker, C. Kirschbaum, M. Marmot and T. Chandola (2010). "Identifying patterns in cortisol secretion in an older population. Findings from the Whitehall II study." Psychoneuroendocrinology **35**(7): 1091-1099.

Kumari, M., M. Shipley, M. Stafford and M. Kivimaki (2011). "Association of diurnal patterns in salivary cortisol with all-cause and cardiovascular mortality: findings from the Whitehall II study." The Journal of clinical endocrinology and metabolism **96**(5): 1478-1485.

Kupper, N., E. J. de Geus, M. van den Berg, C. Kirschbaum, D. I. Boomsma and G. Willemsen (2005). "Familial influences on basal salivary cortisol in an adult population." Psychoneuroendocrinology **30**(9): 857-868.

Lachin, J. M., S. Genuth, D. M. Nathan, B. Zinman and B. N. Rutledge (2008). "Effect of glycemic exposure on the risk of microvascular complications in the diabetes control and complications trial--revisited." Diabetes **57**(4): 995-1001.

Langendam, M., Y. M. Luijf, L. Hooft, J. H. Devries, A. H. Mudde and R. J. Scholten (2012). "Continuous glucose monitoring systems for type 1 diabetes mellitus." Cochrane database of systematic reviews **1**: CD008101.

Larsson, C. A., B. Gullberg, L. Rastam and U. Lindblad (2009). "Salivary cortisol differs with age and sex and shows inverse associations with WHR in Swedish women: a cross-sectional study." BMC Endocr Disord **9**: 16.

Lawton, J., D. Rankin, J. Elliott, S. R. Heller, H. A. Rogers, N. De Zoysa, S. Amiel and U. K. N. D. S. Group (2014). "Experiences, views, and support needs of family members of people with hypoglycemia unawareness: interview study." Diabetes Care **37**(1): 109-115.

Lederbogen, F., C. Kuhner, C. Kirschbaum, C. Meisinger, J. Lammich, R. Holle, B. Krumm, T. von Lengerke, H. E. Wichmann, M. Deuschle and K. H. Ladwig (2010). "Salivary cortisol in a middle-aged community sample: results from 990 men and women of the KORA-F3 Augsburg study." European journal of endocrinology / European Federation of Endocrine Societies **163**(3): 443-451.

Lee, A. S., B. A. Brooks, L. Simmons, M. J. Kilborn, J. Wong, S. M. Twigg and D. K. Yue (2016). "Hypoglycaemia and QT interval prolongation: Detection by simultaneous Holter and continuous glucose monitoring." Diabetes Res Clin Pract.

Lee, D. Y., E. Kim and M. H. Choi (2015). "Technical and clinical aspects of cortisol as a biochemical marker of chronic stress." BMB Rep.

Lee, J. G., I. S. Choi, E. J. Park, J. H. Cho, M. G. Lee, B. J. Choi and I. S. Jang (2007). "beta(2)-Adrenoceptor-mediated facilitation of glutamatergic transmission in rat ventromedial hypothalamic neurons." Neuroscience **144**(4): 1255-1265.

Lee, Y., M. Y. Wang, X. Q. Du, M. J. Charron and R. H. Unger (2011). "Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice." Diabetes **60**(2): 391-397.

Lee, Z. S., J. C. Chan, V. T. Yeung, C. C. Chow, M. S. Lau, G. T. Ko, J. K. Li, C. S. Cockram and J. A. Critchley (1999). "Plasma insulin, growth hormone, cortisol, and central obesity among young Chinese type 2 diabetic patients." Diabetes care **22**(9): 1450-1457.

Leelarathna, L., S. A. Little, E. Walkinshaw, H. K. Tan, A. Lubina-Solomon, K. Kumareswaran, A. P. Lane, T. Chadwick, S. M. Marshall, J. Speight, D. Flanagan, S. R. Heller, J. A. Shaw and M. L. Evans (2013). "Restoration of Self-awareness of Hypoglycemia in Adults With Long-standing Type 1 Diabetes: Hyperinsulinemic-hypoglycemic clamp substudy results from the HypoCOMPaSS trial." Diabetes care.

Leelarathna, L., S. A. Little, E. Walkinshaw, H. K. Tan, A. Lubina-Solomon, K. Kumareswaran, A. P. Lane, T. Chadwick, S. M. Marshall, J. Speight, D. Flanagan, S. R. Heller, J. A. Shaw and M. L. Evans (2013). "Restoration of self-awareness of hypoglycemia in adults with long-standing type 1 diabetes: hyperinsulinemic-hypoglycemic clamp substudy results from the HypoCOMPaSS trial." Diabetes Care **36**(12): 4063-4070.

Leelarathna, L., M. Nodale, J. M. Allen, D. Elleri, K. Kumareswaran, A. Haidar, K. Caldwell, M. E. Wilinska, C. L. Acerini, M. L. Evans, H. R. Murphy, D. B. Dunger and R. Hovorka (2013). "Evaluating

the accuracy and large inaccuracy of two continuous glucose monitoring systems." Diabetes technology & therapeutics **15**(2): 143-149.

Levi, F. and U. Schibler (2007). "Circadian rhythms: mechanisms and therapeutic implications." Annu Rev Pharmacol Toxicol **47**: 593-628.

Lewis, J. G. (2006). "Steroid analysis in saliva: an overview." Clin Biochem Rev **27**(3): 139-146.

Li, W., X. Liu, M. Yanoff, S. Cohen and X. Ye (1996). "Cultured retinal capillary pericytes die by apoptosis after an abrupt fluctuation from high to low glucose levels: a comparative study with retinal capillary endothelial cells." Diabetologia **39**(5): 537-547.

Libby, P., P. M. Ridker and A. Maseri (2002). "Inflammation and atherosclerosis." Circulation **105**(9): 1135-1143.

Licinio, J., M. L. Wong and P. W. Gold (1996). "The hypothalamic-pituitary-adrenal axis in anorexia nervosa." Psychiatry research **62**(1): 75-83.

Lightman, S. L., C. C. Wiles, H. C. Atkinson, D. E. Henley, G. M. Russell, J. A. Leendertz, M. A. McKenna, F. Spiga, S. A. Wood and B. L. Conway-Campbell (2008). "The significance of glucocorticoid pulsatility." European journal of pharmacology **583**(2-3): 255-262.

Little, S., T. Chadwick, P. Choudhary, C. Brennand, J. Stickland, S. Barendse, T. Olateju, L. Leelarathna, E. Walkinshaw, H. K. Tan, S. M. Marshall, R. M. Thomas, S. Heller, M. Evans, D. Kerr, D. Flanagan, J. Speight and J. A. Shaw (2012). "Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglycaemia (the Hypo COMPaSS trial)." BMC endocrine disorders **12**: 33.

Liu, D., J. Diorio, B. Tannenbaum, C. Caldji, D. Francis, A. Freedman, S. Sharma, D. Pearson, P. M. Plotsky and M. J. Meaney (1997). "Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress." Science **277**(5332): 1659-1662.

Liu, D., E. Moberg, M. Kollind, P. E. Lins, U. Adamson and I. A. Macdonald (1992). "Arterial, arterialized venous, venous and capillary blood glucose measurements in normal man during hyperinsulinaemic euglycaemia and hypoglycaemia." Diabetologia **35**(3): 287-290.

Liu, H., D. M. Bravata, J. Cabaccan, H. Raff and E. Ryzen (2005). "Elevated late-night salivary cortisol levels in elderly male type 2 diabetic veterans." Clinical endocrinology **63**(6): 642-649.

Liu, J. H., R. R. Kazer and D. D. Rasmussen (1987). "Characterization of the twenty-four hour secretion patterns of adrenocorticotropin and cortisol in normal women and patients with Cushing's disease." The Journal of clinical endocrinology and metabolism **64**(5): 1027-1035.

Luger, A., P. A. Deuster, S. B. Kyle, W. T. Gallucci, L. C. Montgomery, P. W. Gold, D. L. Loriaux and G. P. Chrousos (1987). "Acute hypothalamic-pituitary-adrenal responses to the stress of treadmill exercise. Physiologic adaptations to physical training." The New England journal of medicine **316**(21): 1309-1315.

Luscher, T. F. (1990). "Endothelium-derived vasoactive factors and regulation of vascular tone in human blood vessels." Lung **168 Suppl**: 27-34.

Ly, T. T., J. Hewitt, R. J. Davey, E. M. Lim, E. A. Davis and T. W. Jones (2011). "Improving epinephrine responses in hypoglycemia unawareness with real-time continuous glucose monitoring in adolescents with type 1 diabetes." Diabetes care **34**(1): 50-52.

Ly, T. T., J. A. Nicholas, A. Retterath, E. M. Lim, E. A. Davis and T. W. Jones (2013). "Effect of sensor-augmented insulin pump therapy and automated insulin suspension vs standard insulin pump therapy on hypoglycemia in patients with type 1 diabetes: a randomized clinical trial." JAMA **310**(12): 1240-1247.

Ly, T. T., J. A. Nicholas, A. Retterath, E. M. Lim, E. A. Davis and T. W. Jones (2013). "Effect of sensor-augmented insulin pump therapy and automated insulin suspension vs standard insulin pump therapy on hypoglycemia in patients with type 1 diabetes: a randomized clinical trial." JAMA : the journal of the American Medical Association **310**(12): 1240-1247.

M'Bemba, J., L. Cynober, P. de Bandt, M. Taverna, A. Chevalier, C. Bardin, G. Slama and J. L. Selam (2003). "Effects of dipeptide administration on hypoglycaemic counterregulation in type 1 diabetes." Diabetes & metabolism **29**(4 Pt 1): 412-417.

MacLulich, A. M., I. J. Deary, J. M. Starr, K. J. Ferguson, J. M. Wardlaw and J. R. Seckl (2005). "Plasma cortisol levels, brain volumes and cognition in healthy elderly men." Psychoneuroendocrinology **30**(5): 505-515.

Maeda, S., T. Matsui and S. Yamagishi (2012). "Vildagliptin inhibits oxidative stress and vascular damage in streptozotocin-induced diabetic rats." International journal of cardiology **158**(1): 171-173.

Manenschijn, L., L. Schaap, N. M. van Schoor, S. van der Pas, G. M. Peeters, P. Lips, J. W. Koper and E. F. van Rossum (2013). "High long-term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease." The Journal of clinical endocrinology and metabolism **98**(5): 2078-2083.

Maran, A., J. Lomas, I. A. Macdonald and S. A. Amiel (1995). "Lack of preservation of higher brain function during hypoglycaemia in patients with intensively-treated IDDM." Diabetologia **38**(12): 1412-1418.

Marketou, M. E., A. Kalyva, F. I. Parthenakis, C. Pontikoglou, S. Maragkoudakis, J. E. Kontaraki, G. Chlouverakis, E. A. Zacharis, A. Patrianakos, H. A. Papadaki and P. E. Vardas (2014). "Circulating endothelial progenitor cells in hypertensive patients with increased arterial stiffness." Journal of clinical hypertension **16**(4): 295-300.

Marques, J. L., E. George, S. R. Peacey, N. D. Harris, I. A. Macdonald, T. Cochrane and S. R. Heller (1997). "Altered ventricular repolarization during hypoglycaemia in patients with diabetes." Diabet Med **14**(8): 648-654.

Martin-Timon, I. and F. J. Del Canizo-Gomez (2015). "Mechanisms of hypoglycemia unawareness and implications in diabetic patients." World J Diabetes **6**(7): 912-926.

Martin, S. and R. M. Pangborn (1971). "Human parotid secretion in response to ethyl alcohol." Journal of dental research **50**(2): 485-490.

Marty, N., M. Dallaporta, M. Foretz, M. Emery, D. Tarussio, I. Bady, C. Binnert, F. Beermann and B. Thorens (2005). "Regulation of glucagon secretion by glucose transporter type 2 (glut2) and astrocyte-dependent glucose sensors." The Journal of clinical investigation **115**(12): 3545-3553.

Mason, D. (1991). "Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease." Immunol Today **12**(2): 57-60.

Mathew, R. J. (1986). "Caffeine induced changes in cerebral circulation." Stroke; a journal of cerebral circulation **17**(6): 1337.

Matschinsky, F. M. (1990). "Glucokinase as glucose sensor and metabolic signal generator in pancreatic beta-cells and hepatocytes." Diabetes **39**(6): 647-652.

Matsubara, J., S. Sugiyama, K. Sugamura, T. Nakamura, Y. Fujiwara, E. Akiyama, H. Kurokawa, T. Nozaki, K. Ohba, M. Konishi, H. Maeda, Y. Izumiya, K. Kaikita, H. Sumida, H. Jinnouchi, K. Matsui, S. Kim-Mitsuyama, M. Takeya and H. Ogawa (2012). "A dipeptidyl peptidase-4 inhibitor, des-fluoro-sitagliptin, improves endothelial function and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice." Journal of the American College of Cardiology **59**(3): 265-276.

Matsuura, K., Y. Deyashiki, Y. Bunai, I. Ohya and A. Hara (1996). "Aldose reductase is a major reductase for isocaproaldehyde, a product of side-chain cleavage of cholesterol, in human and animal adrenal glands." Archives of biochemistry and biophysics **328**(2): 265-271.

Matthews, K., J. Schwartz, S. Cohen and T. Seeman (2006). "Diurnal cortisol decline is related to coronary calcification: CARDIA study." Psychosomatic medicine **68**(5): 657-661.

Mayer, J. (1953). "Glucostatic mechanism of regulation of food intake." New England Journal of Medicine **249**: 13-16.

Mazzocchi, G., L. K. Malendowicz, A. Markowska and G. G. Nussdorfer (1994). "Effect of hypophysectomy on corticotropin-releasing hormone and adrenocorticotropin immunoreactivities in the rat adrenal gland." Mol Cell Neurosci **5**(4): 345-349.

McCoy, R. G., H. K. Van Houten, J. Y. Ziegenfuss, N. D. Shah, R. A. Wermers and S. A. Smith (2013). "Self-Report of Hypoglycemia and Health-Related Quality of Life in Patients with Type 1 and Type 2 Diabetes." Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists: 1-28.

McCrimmon, R. (2008). "The mechanisms that underlie glucose sensing during hypoglycaemia in diabetes." Diabetic medicine : a journal of the British Diabetic Association **25**(5): 513-522.

McCrimmon, R. J., I. J. Deary, A. E. Gold, D. A. Hepburn, K. M. MacLeod, F. M. Ewing and B. M. Frier (2003). "Symptoms reported during experimental hypoglycaemia: effect of method of induction of hypoglycaemia and of diabetes per se." Diabetic medicine : a journal of the British Diabetic Association **20**(6): 507-509.

McCrimmon, R. J., M. L. Evans, X. Fan, E. C. McNay, O. Chan, Y. Ding, W. Zhu, D. X. Gram and R. S. Sherwin (2005). "Activation of ATP-sensitive K⁺ channels in the ventromedial hypothalamus amplifies counterregulatory hormone responses to hypoglycemia in normal and recurrently hypoglycemic rats." Diabetes **54**(11): 3169-3174.

McCrimmon, R. J., M. L. Evans, X. Fan, E. C. McNay, O. Chan, Y. Ding, W. Zhu, D. X. Gram and R. S. Sherwin (2005). "Activation of ATP-sensitive potassium channels in the ventromedial hypothalamus amplifies counterregulatory responses to acute hypoglycemia in normal and recurrently hypoglycemic rats." Diabetes **54**: (in press).

McCrimmon, R. J., B. M. Frier and I. J. Deary (1999). "Appraisal of mood and personality during hypoglycaemia in human subjects." Physiol Behav **67**(1): 27-33.

McCrimmon, R. J., C. M. Ryan and B. M. Frier (2012). "Diabetes and cognitive dysfunction." Lancet **379**(9833): 2291-2299.

McCrimmon, R. J., M. Shaw, X. Fan, H. Cheng, Y. Ding, M. C. Vella, L. Zhou, E. C. McNay and R. S. Sherwin (2008). "Key role for AMP-activated protein kinase in the ventromedial hypothalamus in regulating counterregulatory hormone responses to acute hypoglycemia." Diabetes **57**(2): 444-450.

McCrimmon, R. J. and R. S. Sherwin (2010). "Hypoglycemia in type 1 diabetes." Diabetes **59**(10): 2333-2339.

McCrimmon, R. J., Z. Song, H. Cheng, E. C. McNay, C. Weikart-Yeckel, X. Fan, V. H. Routh and R. S. Sherwin (2006). "Corticotrophin-releasing factor receptors within the ventromedial hypothalamus regulate hypoglycemia-induced hormonal counterregulation." J Clin Invest **116**(6): 1723-1730.

McCrimmon, R. J., Z. Song, H. Cheng, E. C. McNay, C. Weikart-Yeckel, X. Fan, V. H. Routh and R. S. Sherwin (2006). "Corticotrophin-releasing factor receptors within the ventromedial hypothalamus regulate hypoglycemia-induced hormonal counterregulation." The Journal of clinical investigation **116**(6): 1723-1730.

McEwen, B. S., D. Albeck, H. Cameron, H. M. Chao, E. Gould, N. Hastings, Y. Kuroda, V. Luine, A. M. Magarinos, C. R. McKittrick and et al. (1995). "Stress and the brain: a paradoxical role for adrenal steroids." Vitamins and hormones **51**: 371-402.

McEwen, B. S. and A. M. Magarinos (1997). "Stress effects on morphology and function of the hippocampus." Annals of the New York Academy of Sciences **821**: 271-284.

McEwen, B. S. and T. Seeman (1999). "Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load." Annals of the New York Academy of Sciences **896**: 30-47.

McGuire, E. A., J. H. Helderman, J. D. Tobin, R. Andres and M. Berman (1976). "Effects of arterial versus venous sampling on analysis of glucose kinetics in man." J Appl Physiol **41**(4): 565-573.

McNicol, A. M., M. A. Kubba and E. McTeague (1988). "The mitogenic effects of corticotrophin-releasing factor on the anterior pituitary gland of the rat." The Journal of endocrinology **118**(2): 237-241.

Menegazzo, L., M. Albiero, A. Avogaro and G. P. Fadini (2012). "Endothelial progenitor cells in diabetes mellitus." BioFactors **38**(3): 194-202.

Merl, V., A. Peters, K. M. Oltmanns, W. Kern, C. Hubold, H. L. Fehm, J. Born and B. Schultes (2005). "Antecedent hypoglycaemia attenuates vascular endothelial growth factor response to subsequent hypoglycaemia in healthy men." Diabetic medicine : a journal of the British Diabetic Association **22**(9): 1278-1281.

Meulenberg, P. M. and J. A. Hofman (1990). "The effect of oral contraceptive use and pregnancy on the daily rhythm of cortisol and cortisone." Clinica chimica acta; international journal of clinical chemistry **190**(3): 211-221.

Miki, T., B. Liss, K. Minami, T. Shiuchi, A. Saraya, Y. Kashima, M. Horiuchi, F. Ashcroft, Y. Minokoshi, J. Roeper and S. Seino (2001). "ATP-sensitive K⁺ channels in the hypothalamus are essential for the maintenance of glucose homeostasis." Nat Neurosci **4**(5): 507-512.

Miki, T., B. Liss, K. Minami, T. Shiuchi, A. Saraya, Y. Kashima, M. Horiuchi, F. Ashcroft, Y. Minokoshi, J. Roeper and S. Seino (2001). "ATP-sensitive K⁺ channels in the hypothalamus are essential for the maintenance of glucose homeostasis." Nature neuroscience **4**(5): 507-512.

Miki, T., B. Liss, K. Minami, T. Shiuchi, A. Saraya, Y. Kashima, M. Horiuchi, F. Ashcroft, Y. Minokoshi, J. Roeper and S. Seino (2001). "ATP-sensitive K⁺ channels in the hypothalamus are essential for the maintenance of glucose homeostasis.[see comment]." Nature Neuroscience **4**(5): 507-512.

Miller, G. E., E. Chen and E. S. Zhou (2007). "If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans." Psychol Bull **133**(1): 25-45.

Milman, S., J. Leu, H. Shamoon, S. Vele and I. Gabriely (2012). "Magnitude of exercise-induced beta-endorphin response is associated with subsequent development of altered hypoglycemia counterregulation." The Journal of clinical endocrinology and metabolism **97**(2): 623-631.

Mitrakou, A., C. Fanelli, T. Veneman, G. Perriello, S. Calderone, D. Platanisiotis, A. Rambotti, S. Raptis, P. Brunetti, P. Cryer and et al. (1993). "Reversibility of unawareness of hypoglycemia in patients with insulinomas." The New England journal of medicine **329**(12): 834-839.

Mitrakou, A., C. Ryan, T. Veneman, M. Moka, T. Jenssen, I. Kiss, J. Durrant, P. Cryer and J. Gerich (1991). "Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction." Am J Physiol **260**(1 Pt 1): E67-74.

Mitrakou, A., C. Ryan, T. Veneman, M. Moka, T. Jenssen, I. Kiss, J. Durrant, P. Cryer and J. Gerich (1991). "Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction." The American journal of physiology **260**(1 Pt 1): E67-74.

Moen, M. F., M. Zhan, V. D. Hsu, L. D. Walker, L. M. Einhorn, S. L. Seliger and J. C. Fink (2009). "Frequency of hypoglycemia and its significance in chronic kidney disease." Clin J Am Soc Nephrol **4**(6): 1121-1127.

Moheet, A., A. Kumar, L. E. Eberly, J. Kim, R. Roberts and E. R. Seaquist (2014). "Hypoglycemia-associated autonomic failure in healthy humans: comparison of two vs three periods of hypoglycemia on hypoglycemia-induced counterregulatory and symptom response 5 days later." The Journal of clinical endocrinology and metabolism **99**(2): 664-670.

Moka, M., A. Mitrakou, T. Veneman, C. Ryan, M. Korytkowski, P. Cryer and J. Gerich (1994). "Hypoglycemia unawareness in IDDM." Diabetes care **17**(12): 1397-1403.

Monnier, L., C. Colette, E. Mas, F. Michel, J. P. Cristol, C. Boegner and D. R. Owens (2010). "Regulation of oxidative stress by glycaemic control: evidence for an independent inhibitory effect of insulin therapy." Diabetologia **53**(3): 562-571.

Monnier, L., C. Colette and D. Owens (2012). "The glycemic triumvirate and diabetic complications: is the whole greater than the sum of its component parts?" Diabetes research and clinical practice **95**(3): 303-311.

Monnier, L., E. Mas, C. Ginet, F. Michel, L. Villon, J. P. Cristol and C. Colette (2006). "Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes." JAMA : the journal of the American Medical Association **295**(14): 1681-1687.

Monnier, L. H., H. Lachkar, J. L. Richard, C. Colette, D. Borgel, A. Orsetti and J. Mirouze (1984). "Plasma beta-thromboglobulin response to insulin-induced hypoglycemia in type I diabetic patients." Diabetes **33**(9): 907-909.

Moore, R. Y. and V. B. Eichler (1972). "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain research **42**(1): 201-206.

Mortensen, E. L., H. H. Jensen, S. A. Sanders and J. M. Reinisch (2006). "Associations between volume of alcohol consumption and social status, intelligence, and personality in a sample of young adult Danes." Scand J Psychol **47**(5): 387-398.

Mosenzon, O., I. Raz, B. M. Scirica, B. Hirshberg, C. I. Stahre, P. G. Steg, J. Davidson, P. Ohman, D. L. Price, B. Frederich, J. A. Udell, E. Braunwald and D. L. Bhatt (2013). "Baseline characteristics of the patient population in the Saxagliptin Assessment of Vascular Outcomes Recorded in patients with diabetes mellitus (SAVOR)-TIMI 53 trial." Diabetes/metabolism research and reviews **29**(5): 417-426.

Mudaliar, S. and R. R. Henry (2010). "Effects of incretin hormones on beta-cell mass and function, body weight, and hepatic and myocardial function." The American journal of medicine **123**(3 Suppl): S19-27.

Muggeo, M., G. Zoppini, E. Bonora, E. Brun, R. C. Bonadonna, P. Moghetti and G. Verlato (2000). "Fasting plasma glucose variability predicts 10-year survival of type 2 diabetic patients: the Verona Diabetes Study." Diabetes care **23**(1): 45-50.

Muller, M. B., S. Zimmermann, I. Sillaber, T. P. Hagemeyer, J. M. Deussing, P. Timpl, M. S. Kormann, S. K. Droste, R. Kuhn, J. M. Reul, F. Holsboer and W. Wurst (2003). "Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress." Nature neuroscience **6**(10): 1100-1107.

Munck, A. and A. Naray-Fejes-Toth (1992). "The ups and downs of glucocorticoid physiology. Permissive and suppressive effects revisited." Molecular and cellular endocrinology **90**(1): C1-4.

Munding, T. O., Q. Mei, D. P. Figlewicz, A. Lernmark and G. J. Taborsky, Jr. (2003). "Impaired glucagon response to sympathetic nerve stimulation in the BB diabetic rat: effect of early sympathetic islet neuropathy." Am J Physiol Endocrinol Metab **285**(5): E1047-1054.

Murphy, B. A., K. A. Fakira, Z. Song, A. Beuve and V. H. Routh (2009). "AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons." American journal of physiology. Cell physiology **297**(3): C750-758.

Murphy, B. A., K. A. Fakira, Z. Song, A. Beuve and V. H. Routh (2009). "AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons." Am J Physiol Cell Physiol **297**(3): C750-758.

Nalla, A. A., G. Thomsen, G. M. Knudsen and V. G. Frokjaer (2015). "The effect of storage conditions on salivary cortisol concentrations using an Enzyme Immunoassay." Scand J Clin Lab Invest **75**(1): 92-95.

Nalysnyk, L., M. Hernandez-Medina and G. Krishnarajah (2010). "Glycaemic variability and complications in patients with diabetes mellitus: evidence from a systematic review of the literature." Diabetes, obesity & metabolism **12**(4): 288-298.

Nathan, D. M., P. A. Cleary, J. Y. Backlund, S. M. Genuth, J. M. Lachin, T. J. Orchard, P. Raskin and B. Zinman (2005). "Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes." The New England journal of medicine **353**(25): 2643-2653.

Nathan, D. M., J. Kuenen, R. Borg, H. Zheng, D. Schoenfeld and R. J. Heine (2008). "Translating the A1C assay into estimated average glucose values." Diabetes care **31**(8): 1473-1478.

Nehlig, A., J. L. Daval and G. Debry (1992). "Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects." Brain Res Brain Res Rev **17**(2): 139-170.

New, J. P., R. Ajjan, A. F. Pfeiffer and G. Freckmann (2015). "Continuous glucose monitoring in people with diabetes: the randomized controlled Glucose Level Awareness in Diabetes Study (GLADIS)." Diabet Med **32**(5): 609-617.

Nicolson, N. A. (2004). "Childhood parental loss and cortisol levels in adult men." Psychoneuroendocrinology **29**(8): 1012-1018.

Nink, M., U. Krause, H. Lehnert and J. Beyer (1991). "Safety and side effects of human and ovine corticotropin-releasing hormone administration in man." Klinische Wochenschrift **69**(5): 185-195.

Noma, A. (1983). "ATP-regulated K⁺ channels in cardiac muscle." Nature **305**(5930): 147-148.

O'Connell, S. M., M. N. Cooper, M. K. Bulsara, E. A. Davis and T. W. Jones (2011). "Reducing rates of severe hypoglycemia in a population-based cohort of children and adolescents with type 1 diabetes over the decade 2000-2009." Diabetes Care **34**(11): 2379-2380.

O'Neill, S. (1997). "Make four the floor." Balance (Diabetes UK)(Jan-Feb): 20-23.

Ogawa, S., M. Ishiki, K. Nako, M. Okamura, M. Senda, T. Mori and S. Ito (2011). "Sitagliptin, a dipeptidyl peptidase-4 inhibitor, decreases systolic blood pressure in Japanese hypertensive patients with type 2 diabetes." The Tohoku journal of experimental medicine **223**(2): 133-135.

Ohno, K. and T. Sakurai (2008). "Orexin neuronal circuitry: role in the regulation of sleep and wakefulness." Frontiers in neuroendocrinology **29**(1): 70-87.

Okamoto, M. M., G. F. Anhe, R. Sabino-Silva, M. F. Marques, H. S. Freitas, R. C. Mori, K. F. Melo and U. F. Machado (2011). "Intensive insulin treatment induces insulin resistance in diabetic rats by impairing glucose metabolism-related mechanisms in muscle and liver." The Journal of endocrinology **211**(1): 55-64.

Okun, M. L., R. T. Krafty, D. J. Buysse, T. H. Monk, C. F. Reynolds, 3rd, A. Begley and M. Hall (2010). "What constitutes too long of a delay? Determining the cortisol awakening response (CAR) using self-report and PSG-assessed wake time." Psychoneuroendocrinology **35**(3): 460-468.

Oldenburg, O., S. D. Critz, M. V. Cohen and J. M. Downey (2003). "Acetylcholine-induced production of reactive oxygen species in adult rabbit ventricular myocytes is dependent on phosphatidylinositol 3- and Src-kinase activation and mitochondrial K(ATP) channel opening." Journal of molecular and cellular cardiology **35**(6): 653-660.

Oltmanns, K. M., B. Dodt, B. Schultes, H. H. Raspe, U. Schweiger, J. Born, H. L. Fehm and A. Peters (2006). "Cortisol correlates with metabolic disturbances in a population study of type 2 diabetic patients." European journal of endocrinology / European Federation of Endocrine Societies **154**(2): 325-331.

Orban, B. O., V. H. Routh, B. E. Levin and J. R. Berlin (2014). "Direct effects of recurrent hypoglycaemia on adrenal catecholamine release." Diab Vasc Dis Res.

Oster, H., S. Damerow, S. Kiessling, V. Jakubcaková, D. Abraham, J. Tian, M. W. Hoffmann and G. Eichele (2006). "The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock." Cell metabolism **4**(2): 163-173.

Otte, C., S. Hart, T. C. Neylan, C. R. Marmar, K. Yaffe and D. C. Mohr (2005). "A meta-analysis of cortisol response to challenge in human aging: importance of gender." Psychoneuroendocrinology **30**(1): 80-91.

Ou, X., H. A. O'Leary and H. E. Broxmeyer (2013). "Implications of DPP4 modification of proteins that regulate stem/progenitor and more mature cell types." Blood **122**(2): 161-169.

Overton, J. M., G. Davis-Gorman and L. A. Fisher (1990). "Central nervous effects of CRF and angiotensin II on cardiac output in conscious rats." J Appl Physiol (1985) **69**(2): 788-791.

Owens, M. J. and C. B. Nemeroff (1991). "Physiology and pharmacology of corticotropin-releasing factor." Pharmacol Rev **43**(4): 425-473.

Oz, G., N. Tesfaye, A. Kumar, D. K. Deelchand, L. E. Eberly and E. R. Seaquist (2011). "Brain glycogen content and metabolism in subjects with type 1 diabetes and hypoglycemia unawareness." Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.

Page, K. A., A. Williamson, N. Yu, E. C. McNay, J. Dzuira, R. J. McCrimmon and R. S. Sherwin (2009). "Medium-chain fatty acids improve cognitive function in intensively treated type 1 diabetic patients and support in vitro synaptic transmission during acute hypoglycemia." Diabetes **58**(5): 1237-1244.

Pak, C., M. Garshasbi, K. Kahrizi, C. Gross, L. H. Apponi, J. J. Noto, S. M. Kelly, S. W. Leung, A. Tzschach, F. Behjati, S. S. Abedini, M. Mohseni, L. R. Jensen, H. Hu, B. Huang, S. N. Stahley, G. Liu, K. R. Williams, S. Burdick, Y. Feng, S. Sanyal, G. J. Bassell, H. H. Ropers, H. Najmabadi, A. H. Corbett, K. H. Moberg and A. W. Kuss (2011). "Mutation of the conserved polyadenosine RNA binding protein, ZC3H14/dNab2, impairs neural function in Drosophila and humans." Proceedings of the National Academy of Sciences of the United States of America **108**(30): 12390-12395.

Papacosta, E. and G. P. Nassis (2011). "Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science." J Sci Med Sport **14**(5): 424-434.

Paramore, D. S., C. G. Fanelli, S. D. Shah and P. E. Cryer (1998). "Forearm norepinephrine spillover during standing, hyperinsulinemia, and hypoglycemia." The American journal of physiology **275**(5 Pt 1): E872-881.

Paranjape, S. A. and K. P. Briski (2005). "Recurrent insulin-induced hypoglycemia causes site-specific patterns of habituation or amplification of CNS neuronal genomic activation." Neuroscience **130**(4): 957-970.

Paranjape, S. A., O. Chan, W. Zhu, A. M. Horblitt, E. C. McNay, J. A. Cresswell, J. S. Bogan, R. J. McCrimmon and R. S. Sherwin (2010). "Influence of insulin in the ventromedial hypothalamus on pancreatic glucagon secretion in vivo." Diabetes **59**(6): 1521-1527.

Pardal, R. and J. Lopez-Barneo (2002). "Low glucose-sensing cells in the carotid body." Nature neuroscience **5**(3): 197-198.

Parton, L. E., C. P. Ye, R. Coppari, P. J. Enriori, B. Choi, C. Y. Zhang, C. Xu, C. R. Vianna, N. Balthasar, C. E. Lee, J. K. Elmquist, M. A. Cowley and B. B. Lowell (2007). "Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity." Nature **449**(7159): 228-232.

Pasquali, R., S. Cantobelli, F. Casimirri, M. Capelli, L. Bortoluzzi, R. Flaminia, A. M. Labate and L. Barbara (1993). "The hypothalamic-pituitary-adrenal axis in obese women with different patterns of body fat distribution." The Journal of clinical endocrinology and metabolism **77**(2): 341-346.

Patel, A., S. MacMahon, J. Chalmers, B. Neal, L. Billot, M. Woodward, M. Marre, M. Cooper, P. Glasziou, D. Grobbee, P. Hamet, S. Harrap, S. Heller, L. Liu, G. Mancia, C. E. Mogensen, C. Pan, N. Poulter, A. Rodgers, B. Williams, S. Bompont, B. E. de Galan, R. Joshi and F. Travert (2008). "Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes." The New England journal of medicine **358**(24): 2560-2572.

Patterson, C. C., G. Dahlquist, V. Harjutsalo, G. Joner, R. G. Feltbower, J. Svensson, E. Schober, E. Gyurus, C. Castell, B. Urbonaite, J. Rosenbauer, V. Iotova, A. V. Thorsson and G. Soltesz (2007). "Early mortality in EURODIAB population-based cohorts of type 1 diabetes diagnosed in childhood since 1989." Diabetologia **50**(12): 2439-2442.

Paty, B. W., P. A. Senior, J. R. Lakey, A. M. Shapiro and E. A. Ryan (2006). "Assessment of glycemic control after islet transplantation using the continuous glucose monitor in insulin-independent versus insulin-requiring type 1 diabetes subjects." Diabetes technology & therapeutics **8**(2): 165-173.

Pearson, R. M. (1977). "Pharmacokinetics and response to diazoxide in renal failure." Clinical pharmacokinetics **2**(3): 198-204.

Pearson, R. M. (1977). "Pharmacokinetics and response to diazoxide in renal failure." Clin Pharmacokinetics **2**(3): 198-204.

Pedersen-Bjergaard, U., T. Hoi-Hansen and B. Thorsteinsson (2007). "An evaluation of methods of assessing impaired awareness of hypoglycemia in type 1 diabetes: response to Geddes et al." Diabetes care **30**(11): e112; author reply e113.

Pedersen-Bjergaard, U., S. Pramming, S. R. Heller, T. M. Wallace, A. K. Rasmussen, H. V. Jorgensen, D. R. Matthews, P. Hougaard and B. Thorsteinsson (2004). "Severe hypoglycaemia in 1076 adult patients with type 1 diabetes: influence of risk markers and selection." Diabetes/metabolism research and reviews **20**(6): 479-486.

Peeters, F., N. A. Nicolson and J. Berkhof (2004). "Levels and variability of daily life cortisol secretion in major depression." Psychiatry research **126**(1): 1-13.

Pende, A., N. R. Musso, C. Vergassola, A. Ioverno, G. Galbariggi and G. Lotti (1991). "Absence of correlations between plasma catecholamine levels and mononuclear leukocyte beta 2-adrenergic receptors in the elderly." Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie **45**(9): 383-386.

Penhoat, A., P. G. Chatelain, C. Jaillard and J. M. Saez (1988). "Characterization of insulin-like growth factor I and insulin receptors on cultured bovine adrenal fasciculata cells. Role of these peptides on adrenal cell function." Endocrinology **122**(6): 2518-2526.

Pepper, G. M., J. Steinsapir and K. Reynolds (2012). "Effect of short-term iPRO continuous glucose monitoring on hemoglobin A1c levels in clinical practice." Diabetes technology & therapeutics **14**(8): 654-657.

Perantie, D. C., A. Lim, J. Wu, P. Weaver, S. L. Warren, M. Sadler, N. H. White and T. Hershey (2008). "Effects of prior hypoglycemia and hyperglycemia on cognition in children with type 1 diabetes mellitus." Pediatric diabetes **9**(2): 87-95.

Perkins, B. A., D. Z. Cherney, H. Partridge, N. Soleymanlou, H. Tschirhart, B. Zinman, N. M. Fagan, S. Kaspers, H. J. Woerle, U. C. Broedl and O. E. Johansen (2014). "Sodium-Glucose Cotransporter 2 Inhibition and Glycemic Control in Type 1 Diabetes: Results of an 8-Week Open-Label Proof-of-Concept Trial." Diabetes care **37**(5): 1480-1483.

Peters, A., F. Rohloff and W. Kerner (1995). "Preserved counterregulatory hormone release and symptoms after short term hypoglycemic episodes in normal men." The Journal of clinical endocrinology and metabolism **80**(10): 2894-2898.

Pickup, J. C., S. C. Freeman and A. J. Sutton (2011). "Glycaemic control in type 1 diabetes during real time continuous glucose monitoring compared with self monitoring of blood glucose: meta-analysis of randomised controlled trials using individual patient data." BMJ **343**: d3805.

Pickup, J. C. and A. J. Sutton (2008). "Severe hypoglycaemia and glycaemic control in Type 1 diabetes: meta-analysis of multiple daily insulin injections compared with continuous subcutaneous insulin infusion." Diabetic medicine : a journal of the British Diabetic Association **25**(7): 765-774.

Pincus, G. (1943). "A diurnal rhythm in the excretion of urinary ketosteroids by young men." Journal of clinical endocrinology **3**: 195-199.

Pitts, A. F., S. D. Samuelson, W. H. Meller, G. Bissette, C. B. Nemeroff and R. G. Kathol (1995). "Cerebrospinal fluid corticotropin-releasing hormone, vasopressin, and oxytocin concentrations in treated patients with major depression and controls." Biological psychiatry **38**(5): 330-335.

Plotsky, P. M., T. O. Bruhn and W. Vale (1985). "Hypophysiotropic regulation of adrenocorticotropin secretion in response to insulin-induced hypoglycemia." Endocrinology **117**(1): 323-329.

Plum, L., X. Ma, B. Hampel, N. Balthasar, R. Coppari, H. Munzberg, M. Shanabrough, D. Burdakov, E. Rother, R. Janoschek, J. Alber, B. F. Belgardt, L. Koch, J. Seibler, F. Schwenk, C. Fekete, A. Suzuki, T. W. Mak, W. Krone, T. L. Horvath, F. M. Ashcroft and J. C. Bruning (2006). "Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity." The Journal of clinical investigation **116**(7): 1886-1901.

Pocai, A., T. K. Lam, R. Gutierrez-Juarez, S. Obici, G. J. Schwartz, J. Bryan, L. Aguilar-Bryan and L. Rossetti (2005). "Hypothalamic K(ATP) channels control hepatic glucose production." Nature **434**(7036): 1026-1031.

Porcellati, F., S. Pampanelli, P. Rossetti, N. Busciantella Ricci, S. Marzotti, P. Lucidi, F. Santeusano, G. B. Bolli and C. G. Fanelli (2007). "Effect of the amino acid alanine on glucagon secretion in non-diabetic and type 1 diabetic subjects during hyperinsulinaemic euglycaemia, hypoglycaemia and post-hypoglycaemic hyperglycaemia." Diabetologia **50**(2): 422-430.

Porksen, S., L. B. Nielsen, A. Kaas, M. Kocova, F. Chiarelli, C. Orskov, J. J. Holst, K. B. Ploug, P. Hougaard, L. Hansen and H. B. Mortensen (2007). "Meal-stimulated glucagon release is associated with postprandial blood glucose level and does not interfere with glycemic control in children and adolescents with new-onset type 1 diabetes." The Journal of clinical endocrinology and metabolism **92**(8): 2910-2916.

Porte, D., Jr., S. C. Woods, M. Chen, P. H. Smith and J. W. Ensink (1975). "Central factors in the control of insulin and glucagon secretion." Pharmacol Biochem Behav **3**(1 Suppl): 127-133.

Powell, A. M., R. S. Sherwin and G. I. Shulman (1993). "Impaired hormonal responses to hypoglycemia in spontaneously diabetic and recurrently hypoglycemic rats. Reversibility and stimulus specificity of the deficits." The Journal of clinical investigation **92**(6): 2667-2674.

Powell, L. H., W. R. Lavallo, K. A. Matthews, P. Meyer, A. R. Midgley, A. Baum, A. A. Stone, L. Underwood, J. J. McCann, K. Janikula Herro and M. G. Ory (2002). "Physiologic markers of chronic stress in premenopausal, middle-aged women." Psychosomatic medicine **64**(3): 502-509.

Power, C., L. Li and C. Hertzman (2008). "Cognitive development and cortisol patterns in mid-life: findings from a British birth cohort." Psychoneuroendocrinology **33**(4): 530-539.

Pramming, S., B. Thorsteinsson, I. Bendtson and C. Binder (1990). "The relationship between symptomatic and biochemical hypoglycaemia in insulin-dependent diabetic patients." J Intern Med **228**(6): 641-646.

Pramming, S., B. Thorsteinsson, I. Bendtson and C. Binder (1991). "Symptomatic hypoglycaemia in 411 type 1 diabetic patients." Diabetic medicine : a journal of the British Diabetic Association **8**(3): 217-222.

Proost, P., S. Struyf, D. Schols, C. Durinx, A. Wuyts, J. P. Lenaerts, E. De Clercq, I. De Meester and J. Van Damme (1998). "Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha." FEBS letters **432**(1-2): 73-76.

Pruessner, J. C., C. Kirschbaum, G. Meinlschmid and D. H. Hellhammer (2003). "Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change." Psychoneuroendocrinology **28**(7): 916-931.

Pruessner, J. C., O. T. Wolf, D. H. Hellhammer, A. Buske-Kirschbaum, K. von Auer, S. Jobst, F. Kaspers and C. Kirschbaum (1997). "Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity." Life sciences **61**(26): 2539-2549.

Puente, E., Daphana-Iken D, Bree AJ (2009). Impaired Counterregulatory response to hypoglycaemia and impaired glucose tolerance in brain glucose transporter 4 (GLUT4) knockout mice, Diabetes.

Putignano, P., A. Dubini, P. Toja, C. Invitti, S. Bonfanti, G. Redaelli, D. Zappulli and F. Cavagnini (2001). "Salivary cortisol measurement in normal-weight, obese and anorexic women: comparison with plasma cortisol." European journal of endocrinology / European Federation of Endocrine Societies **145**(2): 165-171.

Quagliaro, L., L. Piconi, R. Assaloni, L. Martinelli, E. Motz and A. Ceriello (2003). "Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation." Diabetes **52**(11): 2795-2804.

Qvigstad, E., M. Kollind and V. Grill (2004). "Nine weeks of bedtime diazoxide is well tolerated and improves beta-cell function in subjects with Type 2 diabetes." Diabetic medicine : a journal of the British Diabetic Association **21**(1): 73-76.

Raju, B., A. M. Arbelaez, S. M. Breckenridge and P. E. Cryer (2006). "Nocturnal hypoglycemia in type 1 diabetes: an assessment of preventive bedtime treatments." The Journal of clinical endocrinology and metabolism **91**(6): 2087-2092.

Raju, B. and P. E. Cryer (2005). "Loss of the decrement in intraislet insulin plausibly explains loss of the glucagon response to hypoglycemia in insulin-deficient diabetes: documentation of the intraislet insulin hypothesis in humans." Diabetes **54**(3): 757-764.

Raman, V. S., K. J. Mason, L. M. Rodriguez, K. Hassan, X. Yu, L. Bomgaars and R. A. Heptulla (2010). "The role of adjunctive exenatide therapy in pediatric type 1 diabetes." Diabetes care **33**(6): 1294-1296.

Ramanathan, R. and P. E. Cryer (2011). "Adrenergic mediation of hypoglycemia-associated autonomic failure." Diabetes **60**(2): 602-606.

Ramnanan, C. J., D. S. Edgerton, G. Kraft and A. D. Cherrington (2011). "Physiologic action of glucagon on liver glucose metabolism." Diabetes, obesity & metabolism **13 Suppl 1**: 118-125.

Razavi Nematollahi, L., A. E. Kitabchi, F. B. Stentz, J. Y. Wan, B. A. Larijani, M. M. Tehrani, M. H. Gozashti, K. Omidfar and E. Taheri (2009). "Proinflammatory cytokines in response to insulin-induced hypoglycemic stress in healthy subjects." Metabolism: clinical and experimental **58**(4): 443-448.

Reincke, M., B. Allolio, G. Wurth and W. Winkelmann (1993). "The hypothalamic-pituitary-adrenal axis in critical illness: response to dexamethasone and corticotropin-releasing hormone." The Journal of clinical endocrinology and metabolism **77**(1): 151-156.

Reno, C. M., D. Daphna-Iken, S. Chen, J. Vanderweele, K. Jethi and S. J. Fisher (2013). "Severe Hypoglycemia-Induced Lethal Cardiac Arrhythmias Are Mediated by Sympathoadrenal Activation." Diabetes.

Reno, C. M., M. Litvin, A. L. Clark and S. J. Fisher (2013). "Defective counterregulation and hypoglycemia unawareness in diabetes: mechanisms and emerging treatments." Endocrinology and metabolism clinics of North America **42**(1): 15-38.

Revsin, Y. and E. R. de Kloet (2009). "When glucocorticoids change from protective to harmful. Lessons from a type 1 diabetes animal model." Medicina **69**(3): 353-358.

Revsin, Y., D. van Wijk, F. E. Saravia, M. S. Oitzl, A. F. De Nicola and E. R. de Kloet (2008). "Adrenal hypersensitivity precedes chronic hypercorticism in streptozotocin-induced diabetes mice." Endocrinology **149**(7): 3531-3539.

Reynolds, R. M., B. R. Walker, S. Haw, D. E. Newby, D. F. Mackay, S. M. Cobbe, A. C. Pell, C. Fischbacher, S. Pringle, D. Murdoch, F. Dunn, K. Oldroyd, P. Macintyre, B. O'Rourke and J. P. Pell (2010). "Low serum cortisol predicts early death after acute myocardial infarction." Critical care medicine **38**(3): 973-975.

Richardson, T., A. Rozkovec, P. Thomas, J. Ryder, C. Meckes and D. Kerr (2004). "Influence of caffeine on heart rate variability in patients with long-standing type 1 diabetes." Diabetes care **27**(5): 1127-1131.

Ridker, P. M., M. Cushman, M. J. Stampfer, R. P. Tracy and C. H. Hennekens (1997). "Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men." The New England journal of medicine **336**(14): 973-979.

Riedel, M. J., P. Boora, D. Steckley, G. de Vries and P. E. Light (2003). "Kir6.2 polymorphisms sensitize beta-cell ATP-sensitive potassium channels to activation by acyl CoAs: a possible cellular mechanism for increased susceptibility to type 2 diabetes?" Diabetes **52**(10): 2630-2635.

Ringholm, L., U. Pedersen-Bjergaard, B. Thorsteinsson, P. Damm and E. R. Mathiesen (2012). "Hypoglycaemia during pregnancy in women with Type 1 diabetes." Diabetic medicine : a journal of the British Diabetic Association.

Risso, A., F. Mercuri, L. Quagliaro, G. Damante and A. Ceriello (2001). "Intermittent high glucose enhances apoptosis in human umbilical vein endothelial cells in culture." American journal of physiology. Endocrinology and metabolism **281**(5): E924-930.

Ritvanen, T., V. Louhevaara, P. Helin, S. Vaisanen and O. Hanninen (2006). "Responses of the autonomic nervous system during periods of perceived high and low work stress in younger and older female teachers." Appl Ergon **37**(3): 311-318.

Rivier, C. and W. Vale (1983). "Influence of the frequency of ovine corticotropin-releasing factor administration on adrenocorticotropin and corticosterone secretion in the rat." Endocrinology **113**(4): 1422-1426.

Rizza, R. A., P. E. Cryer and J. E. Gerich (1979). "Role of glucagon, catecholamines, and growth hormone in human glucose counterregulation. Effects of somatostatin and combined alpha- and beta-adrenergic blockade on plasma glucose recovery and glucose flux rates after insulin-induced hypoglycemia." The Journal of clinical investigation **64**(1): 62-71.

Rodbard, D. (2011). "Clinical interpretation of indices of quality of glycemic control and glycemic variability." Postgraduate medicine **123**(4): 107-118.

Rohleder, N. and C. Kirschbaum (2006). "The hypothalamic-pituitary-adrenal (HPA) axis in habitual smokers." International journal of psychophysiology : official journal of the International Organization of Psychophysiology **59**(3): 236-243.

Rohlfing, C. L., H. M. Wiedmeyer, R. R. Little, J. D. England, A. Tennill and D. E. Goldstein (2002). "Defining the relationship between plasma glucose and HbA(1c): analysis of glucose profiles and HbA(1c) in the Diabetes Control and Complications Trial." Diabetes care **25**(2): 275-278.

Rosecrans, J. A. and L. D. Karin (1998). "Effects of nicotine on the hypothalamic-pituitary-axis (HPA) and immune function: introduction to the Sixth Nicotine Round Table Satellite, American Society of Addiction Medicine Nicotine Dependence Meeting, November 15, 1997." Psychoneuroendocrinology **23**(2): 95-102.

Rossetti, P., F. Porcellati, N. Busciantella Ricci, P. Candeloro, P. Cioli, K. S. Nair, F. Santeusano, G. B. Bolli and C. G. Fanelli (2008). "Effect of oral amino acids on counterregulatory responses and

cognitive function during insulin-induced hypoglycemia in nondiabetic and type 1 diabetic people." Diabetes **57**(7): 1905-1917.

Roubos, E. W., M. Dahmen, T. Kozicz and L. Xu (2012). "Leptin and the hypothalamo-pituitary-adrenal stress axis." General and comparative endocrinology **177**(1): 28-36.

Routh, V. H. (2002). "Glucose-sensing neurons: are they physiologically relevant?" Physiology & behavior **76**(3): 403-413.

Routh, V. H. (2010). "Glucose sensing neurons in the ventromedial hypothalamus." Sensors (Basel) **10**(10): 9002-9025.

Roy-Byrne, P. P., T. W. Uhde, R. M. Post, W. Gallucci, G. P. Chrousos and P. W. Gold (1986). "The corticotropin-releasing hormone stimulation test in patients with panic disorder." The American journal of psychiatry **143**(7): 896-899.

Roy, M., B. Collier and A. Roy (1990). "Hypothalamic-pituitary-adrenal axis dysregulation among diabetic outpatients." Psychiatry research **31**(1): 31-37.

Roy, M., B. Collier and A. Roy (1991). "Dysregulation of the hypothalamo-pituitary-adrenal axis and duration of diabetes." The Journal of diabetic complications **5**(4): 218-220.

Roy, M. S., A. Roy and S. Brown (1998). "Increased urinary-free cortisol outputs in diabetic patients." Journal of diabetes and its complications **12**(1): 24-27.

Roy, M. S., A. Roy, W. T. Gallucci, B. Collier, K. Young, T. C. Kamilaris and G. P. Chrousos (1993). "The ovine corticotropin-releasing hormone-stimulation test in type I diabetic patients and controls: suggestion of mild chronic hypercortisolism." Metabolism: clinical and experimental **42**(6): 696-700.

Rubin, A. A., F. E. Roth, R. M. Taylor and H. Rosenkilde (1962). "Pharmacology of diazoxide, an antihypertensive, nondiuretic benzothiadiazine." The Journal of pharmacology and experimental therapeutics **136**: 344-352.

Saberi, M., M. Bohland and C. M. Donovan (2008). "The locus for hypoglycemic detection shifts with the rate of fall in glycemia: the role of portal-superior mesenteric vein glucose sensing." Diabetes **57**(5): 1380-1386.

Saisho, Y., C. Tanaka, K. Tanaka, R. Roberts, T. Abe, M. Tanaka, S. Meguro, J. Irie, T. Kawai and H. Itoh (2014). "Relationships among different glycemic variability indices obtained by continuous glucose monitoring." Primary care diabetes.

Samann, A., I. Muhlhauser, R. Bender, C. Kloos and U. A. Muller (2005). "Glycaemic control and severe hypoglycaemia following training in flexible, intensive insulin therapy to enable dietary freedom in people with type 1 diabetes: a prospective implementation study." Diabetologia **48**(10): 1965-1970.

Sanders, N. M., A. A. Dunn-Meynell and B. E. Levin (2004). "Third ventricular alloxan reversibly impairs glucose counterregulatory responses." Diabetes **53**(5): 1230-1236.

Sandoval, D. A., A. C. Ertl, M. A. Richardson, D. B. Tate and S. N. Davis (2003). "Estrogen blunts neuroendocrine and metabolic responses to hypoglycemia." Diabetes **52**(7): 1749-1755.

Sapolsky, R. M., L. C. Krey and B. S. McEwen (1986). "The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis." Endocrine reviews **7**(3): 284-301.

Sarkar, G., M. Alattar, R. J. Brown, M. J. Quon, D. M. Harlan and K. I. Rother (2014). "Exenatide treatment for 6 months improves insulin sensitivity in adults with type 1 diabetes." Diabetes care **37**(3): 666-670.

Sartore, G., N. C. Chilelli, S. Burlina, P. Di Stefano, F. Piarulli, D. Fedele, A. Mosca and A. Lapolla (2012). "The importance of HbA1c and glucose variability in patients with type 1 and type 2 diabetes: outcome of continuous glucose monitoring (CGM)." Acta diabetologica **49 Suppl 1**: S153-160.

Schiavon, M., G. P. Fadini, F. Lunardi, C. Agostini, E. Boscaro, F. Calabrese, G. Marulli and F. Rea (2012). "Increased tissue endothelial progenitor cells in end-stage lung diseases with pulmonary hypertension." The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation **31**(9): 1025-1030.

Schlichtkrull, J., O. Munck and M. Jersild (1965). "The M-Valve, an Index of Blood-Sugar Control in Diabetics." Acta medica Scandinavica **177**: 95-102.

Schuit, F. C., P. Huypens, H. Heimberg and D. G. Pipeleers (2001). "Glucose sensing in pancreatic beta-cells: a model for the study of other glucose-regulated cells in gut, pancreas, and hypothalamus." Diabetes **50**(1): 1-11.

Schulte, H. M., G. P. Chrousos, P. W. Gold, J. D. Booth, E. H. Oldfield, G. B. Cutler, Jr. and D. L. Loriaux (1985). "Continuous administration of synthetic ovine corticotropin-releasing factor in man. Physiological and pathophysiological implications." The Journal of clinical investigation **75**(6): 1781-1785.

Schulte, H. M., G. P. Chrousos, E. H. Oldfield, P. W. Gold, G. B. Cutler and D. L. Loriaux (1985). "Ovine corticotropin-releasing factor administration in normal men. Pituitary and adrenal responses in the morning and evening." Hormone research **21**(2): 69-74.

Schwanstecher, C., U. Meyer and M. Schwanstecher (2002). "K(IR)6.2 polymorphism predisposes to type 2 diabetes by inducing overactivity of pancreatic beta-cell ATP-sensitive K(+) channels." Diabetes **51**(3): 875-879.

Schwartz, N. S., W. E. Clutter, S. D. Shah and P. E. Cryer (1987). "Glycemic thresholds for activation of glucose counterregulatory systems are higher than the threshold for symptoms." The Journal of clinical investigation **79**(3): 777-781.

Scribner, K. A., C. D. Walker, C. S. Cascio and M. F. Dallman (1991). "Chronic streptozotocin diabetes in rats facilitates the acute stress response without altering pituitary or adrenal responsiveness to secretagogues." Endocrinology **129**(1): 99-108.

Seaquist, E. R., J. Anderson, B. Childs, P. Cryer, S. Dagogo-Jack, L. Fish, S. R. Heller, H. Rodriguez, J. Rosenzweig and R. Vigersky (2013). "Hypoglycemia and diabetes: a report of a workgroup of the American Diabetes Association and the Endocrine Society." Diabetes Care **36**(5): 1384-1395.

Seeman, T. E. and R. J. Robbins (1994). "Aging and hypothalamic-pituitary-adrenal response to challenge in humans." Endocrine reviews **15**(2): 233-260.

Segel, S. A., D. S. Paramore and P. E. Cryer (2002). "Hypoglycemia-associated autonomic failure in advanced type 2 diabetes." Diabetes **51**(3): 724-733.

Seok, H., J. H. Huh, H. M. Kim, B. W. Lee, E. S. Kang, H. C. Lee and B. S. Cha (2015). "1,5-anhydroglucitol as a useful marker for assessing short-term glycemic excursions in type 1 diabetes." Diabetes Metab J **39**(2): 164-170.

Service, F. J., G. D. Molnar, J. W. Rosevear, E. Ackerman, L. C. Gatewood and W. F. Taylor (1970). "Mean amplitude of glycemic excursions, a measure of diabetic instability." Diabetes **19**(9): 644-655.

Sesti, G., E. Laratta, M. Cardellini, F. Andreozzi, S. Del Guerra, C. Irace, A. Gnasso, M. Grupillo, R. Lauro, M. L. Hribal, F. Perticone and P. Marchetti (2006). "The E23K variant of KCNJ11 encoding the pancreatic beta-cell adenosine 5'-triphosphate-sensitive potassium channel subunit Kir6.2 is associated with an increased risk of secondary failure to sulfonylurea in patients with type 2 diabetes." J Clin Endocrinol Metab **91**(6): 2334-2339.

Shah, Z., T. Kampfrath, J. A. Deiuliis, J. Zhong, C. Pineda, Z. Ying, X. Xu, B. Lu, S. Moffatt-Bruce, R. Durairaj, Q. Sun, G. Mihai, A. Maiseyeu and S. Rajagopalan (2011). "Long-term dipeptidyl-peptidase 4 inhibition reduces atherosclerosis and inflammation via effects on monocyte recruitment and chemotaxis." Circulation **124**(21): 2338-2349.

Shapiro, A. M., J. R. Lakey, E. A. Ryan, G. S. Korbitt, E. Toth, G. L. Warnock, N. M. Kneteman and R. V. Rajotte (2000). "Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen." The New England journal of medicine **343**(4): 230-238.

Sheikh, H. I., L. R. Dougherty, E. P. Hayden, D. N. Klein and S. M. Singh (2010). "Glucagon-like peptide-1 receptor gene polymorphism (Leu260Phe) is associated with morning cortisol in preschoolers." Progress in neuro-psychopharmacology & biological psychiatry **34**(6): 980-983.

Shi, X. L., Y. Z. Ren and J. Wu (2011). "Intermittent high glucose enhances apoptosis in INS-1 cells." Experimental diabetes research **2011**: 754673.

Shipley, B. A., G. Der, M. D. Taylor and I. J. Deary (2006). "Cognition and all-cause mortality across the entire adult age range: health and lifestyle survey." Psychosom Med **68**(1): 17-24.

Shivers, J. P., L. Mackowiak, H. Anhalt and H. Zisser (2013). "'Turn it off!': diabetes device alarm fatigue considerations for the present and the future." J Diabetes Sci Technol **7**(3): 789-794.

Siafarikas, A., R. J. Johnston, M. K. Bulsara, P. O'Leary, T. W. Jones and E. A. Davis (2012). "Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes." Diabetes Care **35**(8): 1757-1762.

Silver, I. A. and M. Erecinska (1994). "Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals." The Journal of neuroscience : the official journal of the Society for Neuroscience **14**(8): 5068-5076.

Silver, I. A. and M. Erecinska (1994). "Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals." J Neurosci **14**(8): 5068-5076.

Singh, P., A. Jain and G. Kaur (2004). "Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage." Molecular and cellular biochemistry **260**(1-2): 153-159.

Sjostrand, M., N. Iqbal, J. Lu and B. Hirshberg (2014). "Saxagliptin improves glycemic control by modulating postprandial glucagon and C-peptide levels in Chinese patients with type 2 diabetes." Diabetes Res Clin Pract **105**(2): 185-191.

Slomski, A. (2013). "Avoiding hypoglycemia at all costs is crucial for some with diabetes." JAMA : the journal of the American Medical Association **309**(24): 2536-2537.

Smith-Palmer, J., M. Brandle, R. Trevisan, M. Orsini Federici, S. Liabat and W. Valentine (2014). "Assessment of the association between glycemic variability and diabetes-related complications in type 1 and type 2 diabetes." Diabetes research and clinical practice.

Smith, C. B., P. Choudhary, A. Pernet, D. Hopkins and S. A. Amiel (2009). "Hypoglycemia unawareness is associated with reduced adherence to therapeutic decisions in patients with type 1 diabetes: evidence from a clinical audit." Diabetes care **32**(7): 1196-1198.

Smith, D., A. Pernet, J. M. Rosenthal, E. M. Bingham, H. Reid, I. A. Macdonald and S. A. Amiel (2004). "The effect of modafinil on counter-regulatory and cognitive responses to hypoglycaemia." Diabetologia **47**(10): 1704-1711.

Snell-Bergeon, J. K., R. Roman, D. Rodbard, S. Garg, D. M. Maahs, I. E. Schauer, B. C. Bergman, G. L. Kinney and M. Rewers (2010). "Glycaemic variability is associated with coronary artery calcium in men with Type 1 diabetes: the Coronary Artery Calcification in Type 1 Diabetes study." Diabetic medicine : a journal of the British Diabetic Association **27**(12): 1436-1442.

Soedamah-Muthu, S. S., J. H. Fuller, H. E. Mulnier, V. S. Raleigh, R. A. Lawrenson and H. M. Colhoun (2006). "High risk of cardiovascular disease in patients with type 1 diabetes in the U.K.: a cohort study using the general practice research database." Diabetes care **29**(4): 798-804.

Son, G. H., S. Chung and K. Kim (2011). "The adrenal peripheral clock: glucocorticoid and the circadian timing system." Frontiers in neuroendocrinology **32**(4): 451-465.

Song, Z., B. E. Levin, J. J. McArdle, N. Bakhos and V. H. Routh (2001). "Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus." Diabetes **50**(12): 2673-2681.

Song, Z. and V. H. Routh (2005). "Differential effects of glucose and lactate on glucosensing neurons in the ventromedial hypothalamic nucleus." Diabetes **54**(1): 15-22.

Song, Z. and V. H. Routh (2006). "Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamus nucleus." American journal of physiology. Regulatory, integrative and comparative physiology **291**(5): R1283-1287.

Soupal, J., J. Skrha, Jr., M. Fajmon, E. Horova, M. Mraz, J. Skrha and M. Prazny (2014). "Glycemic variability is higher in type 1 diabetes patients with microvascular complications irrespective of glycemic control." Diabetes technology & therapeutics **16**(4): 198-203.

Sprague, J. E. and A. M. Arbelaes (2011). "Glucose counterregulatory responses to hypoglycemia." Pediatr Endocrinol Rev **9**(1): 463-473; quiz 474-465.

Stadelmann, B., L. K. Lin, T. H. Kunz and M. Ruedi (2007). "Molecular phylogeny of New World *Myotis* (Chiroptera, Vespertilionidae) inferred from mitochondrial and nuclear DNA genes." Mol Phylogenet Evol **43**(1): 32-48.

Stagner, J. I., E. Samols and G. C. Weir (1980). "Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration." J Clin Invest **65**(4): 939-942.

Stalder, T., P. Evans, F. Hucklebridge and A. Clow (2010). "Associations between psychosocial state variables and the cortisol awakening response in a single case study." Psychoneuroendocrinology **35**(2): 209-214.

Stalder, T., F. Hucklebridge, P. Evans and A. Clow (2009). "Use of a single case study design to examine state variation in the cortisol awakening response: relationship with time of awakening." Psychoneuroendocrinology **34**(4): 607-614.

Standen, N. B., J. M. Quayle, N. W. Davies, J. E. Brayden, Y. Huang and M. T. Nelson (1989). "Hyperpolarizing vasodilators activate ATP-sensitive K⁺ channels in arterial smooth muscle." Science **245**(4914): 177-180.

Steptoe, A., S. Kunz-Ebrecht, N. Owen, P. J. Feldman, G. Willemssen, C. Kirschbaum and M. Marmot (2003). "Socioeconomic status and stress-related biological responses over the working day." Psychosomatic medicine **65**(3): 461-470.

Steptoe, A., C. H. van Jaarsveld, C. Semmler, R. Plomin and J. Wardle (2009). "Heritability of daytime cortisol levels and cortisol reactivity in children." Psychoneuroendocrinology **34**(2): 273-280.

Stern, C. E., A. M. Owen, I. Tracey, R. B. Look, B. R. Rosen and M. Petrides (2000). "Activity in ventrolateral and mid-dorsolateral prefrontal cortex during nonspatial visual working memory processing: evidence from functional magnetic resonance imaging." Neuroimage **11**(5 Pt 1): 392-399.

Stoddard, S. L., V. K. Bergdall, D. W. Townsend and B. E. Levin (1986). "Plasma catecholamines associated with hypothalamically-elicited defense behavior." Physiology & behavior **36**(5): 867-873.

Stranahan, A. M., T. V. Arumugam, R. G. Cutler, K. Lee, J. M. Egan and M. P. Mattson (2008). "Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons." Nature neuroscience **11**(3): 309-317.

Strand, R., W. Anderson and W. M. Allcroft (1934). "Further studies on the lactic acid, sugar and inorganic phosphorus of the blood of ruminants, (a) following adrenalectomy and (b) after intravenous injection of insulin." The Biochemical journal **28**(2): 642-649.

Stratakis, C. A. and G. P. Chrousos (1995). "Neuroendocrinology and pathophysiology of the stress system." Annals of the New York Academy of Sciences **771**: 1-18.

Stratton, I. M., A. I. Adler, H. A. Neil, D. R. Matthews, S. E. Manley, C. A. Cull, D. Hadden, R. C. Turner and R. R. Holman (2000). "Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study." BMJ **321**(7258): 405-412.

Suda, T., N. Tomori, F. Yajima, E. Odagiri, H. Demura and K. Shizume (1986). "Characterization of immunoreactive corticotropin and corticotropin-releasing factor in human adrenal and ovarian tumours." Acta endocrinologica **111**(4): 546-552.

Suhaimi, F., A. Le Compte, J. C. Preiser, G. M. Shaw, P. Massion, R. Radermecker, C. G. Pretty, J. Lin, T. Desai and J. G. Chase (2010). "What makes tight glycemic control tight? The impact of variability and nutrition in two clinical studies." Journal of diabetes science and technology **4**(2): 284-298.

Symington, T., W. P. Duguid and J. N. Davidson (1956). "Effect of exogenous corticotropin on the histochemical pattern of the human adrenal cortex and a comparison with the changes during stress." The Journal of clinical endocrinology and metabolism **16**(5): 580-598.

Szepietowska, B., W. Zhu, O. Chan, A. Horblitt, J. Dziura and R. S. Sherwin (2011). "Modulation of beta-adrenergic receptors in the ventromedial hypothalamus influences counterregulatory responses to hypoglycemia." Diabetes **60**(12): 3154-3158.

ter Braak, E. W., A. M. Appelman, M. van de Laak, R. P. Stolk, T. W. van Haeften and D. W. Erkelens (2000). "Clinical characteristics of type 1 diabetic patients with and without severe hypoglycemia." Diabetes care **23**(10): 1467-1471.

Thornberry, N. A. and B. Gallwitz (2009). "Mechanism of action of inhibitors of dipeptidyl-peptidase-4 (DPP-4)." Best Pract Res Clin Endocrinol Metab **23**(4): 479-486.

Tie, L., L. Y. Chen, D. D. Chen, H. H. Xie, K. M. Channon and A. F. Chen (2014). "GTP cyclohydrolase I prevents diabetic-impaired endothelial progenitor cells and wound healing by suppressing oxidative stress/thrombospondin-1." American journal of physiology. Endocrinology and metabolism **306**(10): E1120-1131.

Tilders, F. J., F. Berkenbosch and P. G. Smelik (1982). "Adrenergic mechanisms involved in the control of pituitary-adrenal activity in the rat: a beta-adrenergic stimulatory mechanism." Endocrinology **110**(1): 114-120.

Triplitt, C. and E. Chiquette (2006). "Exenatide: from the Gila monster to the pharmacy." Journal of the American Pharmacists Association : JAPhA **46**(1): 44-52; quiz 53-45.

Trollope, A. F., M. Gutierrez-Mecinas, K. R. Mifsud, A. Collins, E. A. Saunderson and J. M. Reul (2012). "Stress, epigenetic control of gene expression and memory formation." Experimental neurology **233**(1): 3-11.

Trovati, M., G. Anfossi, F. Cavalot, S. Vitali, P. Massucco, E. Mularoni, P. Schinco, G. Tamponi and G. Emanuelli (1986). "Studies on mechanisms involved in hypoglycemia-induced platelet activation." Diabetes **35**(7): 818-825.

Trube, G., P. Rorsman and T. Ohno-Shosaku (1986). "Opposite effects of tolbutamide and diazoxide on the ATP-dependent K⁺ channel in mouse pancreatic beta-cells." Pflugers Archiv : European journal of physiology **407**(5): 493-499.

Tupola, S., J. Rajantie and H. K. Akerblom (1998). "Experience of severe hypoglycaemia may influence both patient's and physician's subsequent treatment policy of insulin-dependent diabetes mellitus." Eur J Pediatr **157**(8): 625-627.

van Boekel, G., S. Loves, A. van Sorge, J. Ruinemans-Koerts, T. Rijnders and H. de Boer (2008). "Weight loss in obese men by caloric restriction and high-dose diazoxide-mediated insulin suppression." Diabetes, obesity & metabolism **10**(12): 1195-1203.

Vannucci, S. J., F. Maher and I. A. Simpson (1997). "Glucose transporter proteins in brain: delivery of glucose to neurons and glia." Glia **21**(1): 2-21.

Varanasi, A., N. Bellini, D. Rawal, M. Vora, A. Makdissi, S. Dhindsa, A. Chaudhuri and P. Dandona (2011). "Liraglutide as additional treatment for type 1 diabetes." European journal of endocrinology / European Federation of Endocrine Societies **165**(1): 77-84.

Veldhuis, J. D., A. Iranmanesh, G. Lizarralde and M. L. Johnson (1989). "Amplitude modulation of a burstlike mode of cortisol secretion subserves the circadian glucocorticoid rhythm." The American journal of physiology **257**(1 Pt 1): E6-14.

Vella, S., L. Buetow, P. Royle, S. Livingstone, H. M. Colhoun and J. R. Petrie (2010). "The use of metformin in type 1 diabetes: a systematic review of efficacy." Diabetologia **53**(5): 809-820.

Veneman, T., A. Mitrakou, M. Mookan, P. Cryer and J. Gerich (1993). "Induction of hypoglycemia unawareness by asymptomatic nocturnal hypoglycemia." Diabetes **42**(9): 1233-1237.

Ververs, M. T., C. Rouwe and G. P. Smit (1993). "Complex carbohydrates in the prevention of nocturnal hypoglycaemia in diabetic children." European journal of clinical nutrition **47**(4): 268-273.

Vialard-Miguel, J., N. Belaidi, L. Lembeye and J. B. Corcuff (2005). "Lemon juice alters cortisol assays in saliva." Clinical endocrinology **63**(4): 478-479.

Vindedzis, S. A., B. Marsh, J. L. Sherriff and K. G. Stanton (2013). "Alcohol and type 1 diabetes: Patient knowledge of alcohol-induced sustained hypoglycaemia." Diabetes research and clinical practice **102**(2): e19-20.

Vining, R. F. and R. A. McGinley (1987). "The measurement of hormones in saliva: possibilities and pitfalls." Journal of steroid biochemistry **27**(1-3): 81-94.

Vining, R. F., R. A. McGinley and R. G. Symons (1983). "Hormones in saliva: mode of entry and consequent implications for clinical interpretation." Clinical chemistry **29**(10): 1752-1756.

Vreeburg, S. A., B. P. Kruijtzter, J. van Pelt, R. van Dyck, R. H. DeRijk, W. J. Hoogendijk, J. H. Smit, F. G. Zitman and B. W. Penninx (2009). "Associations between sociodemographic, sampling and health factors and various salivary cortisol indicators in a large sample without psychopathology." Psychoneuroendocrinology **34**(8): 1109-1120.

Walker, J. J., F. Spiga, E. Waite, Z. Zhao, Y. Kershaw, J. R. Terry and S. L. Lightman (2012). "The origin of glucocorticoid hormone oscillations." PLoS Biol **10**(6): e1001341.

Wang, J., A. Alexanian, R. Ying, T. J. Kizhakekuttu, K. Dharmashankar, J. Vasquez-Vivar, D. D. Gutterman and M. E. Widlansky (2012). "Acute exposure to low glucose rapidly induces endothelial dysfunction and mitochondrial oxidative stress: role for AMP kinase." Arteriosclerosis, thrombosis, and vascular biology **32**(3): 712-720.

Wang, R., X. Liu, S. T. Hentges, A. A. Dunn-Meynell, B. E. Levin, W. Wang and V. H. Routh (2004). "The regulation of glucose-excited neurons in the hypothalamic arcuate nucleus by glucose and feeding-relevant peptides." Diabetes **53**(8): 1959-1965.

Watson, J. M., E. J. Jenkins, P. Hamilton, M. J. Lunt and D. Kerr (2000). "Influence of caffeine on the frequency and perception of hypoglycemia in free-living patients with type 1 diabetes." Diabetes care **23**(4): 455-459.

Watson, S. J., J. F. Lopez, E. A. Young, W. Vale, J. Rivier and H. Akil (1988). "Effects of low dose ovine corticotropin-releasing hormone in humans: endocrine relationships and beta-endorphin/beta-lipotropin responses." The Journal of clinical endocrinology and metabolism **66**(1): 10-15.

Watts, A. G. and C. M. Donovan (2010). "Sweet talk in the brain: glucosensing, neural networks, and hypoglycemic counterregulation." Frontiers in neuroendocrinology **31**(1): 32-43.

Wechsler, D. (1981). "Manual for the Wechsler Adult Intelligence Scale Revised. ." New York Psychological Corp.

Weiner, D. K. and N. B. Allen (1991). "Large vessel vasculitis of the central nervous system in systemic lupus erythematosus: report and review of the literature." The Journal of rheumatology **18**(5): 748-751.

Weinstock, R. S., D. Xing, D. M. Maahs, A. Michels, M. R. Rickels, A. L. Peters, R. M. Bergenstal, B. Harris, S. N. Dubose, K. M. Miller and R. W. Beck (2013). "Severe hypoglycemia and diabetic ketoacidosis in adults with type 1 diabetes: results from the T1D Exchange clinic registry." The Journal of clinical endocrinology and metabolism **98**(8): 3411-3419.

Weinstock, R. S., D. Xing, D. M. Maahs, A. Michels, M. R. Rickels, A. L. Peters, R. M. Bergenstal, B. Harris, S. N. Dubose, K. M. Miller, R. W. Beck and T. D. E. C. Network (2013). "Severe hypoglycemia and diabetic ketoacidosis in adults with type 1 diabetes: results from the T1D Exchange clinic registry." J Clin Endocrinol Metab **98**(8): 3411-3419.

Weinstock, R. S., D. Y. Xing, D. M. Maahs, A. Michels, M. R. Rickels, A. L. Peters, R. M. Bergenstal, B. Harris, S. N. DuBose, K. M. Miller, R. W. Beck and T. D. E. C. Network (2013). "Severe Hypoglycemia and Diabetic Ketoacidosis in Adults With Type 1 Diabetes: Results From the T1D Exchange Clinic Registry." Journal of Clinical Endocrinology & Metabolism **98**(8): 3411-3419.

Welsh, J. B., F. R. Kaufman and S. W. Lee (2012). "Accuracy of the Sof-sensor glucose sensor with the iPro calibration algorithm." J Diabetes Sci Technol **6**(2): 475-476.

Welt, C. K., B. T. Kinsley and D. C. Simonson (1998). "Recurrent hypoglycemia does not impair the cortisol response to adrenocorticotropin infusion in healthy humans." Metabolism: clinical and experimental **47**(10): 1252-1257.

Wentholt, I. M., W. Kulik, R. P. Michels, J. B. Hoekstra and J. H. DeVries (2008). "Glucose fluctuations and activation of oxidative stress in patients with type 1 diabetes." Diabetologia **51**(1): 183-190.

Westlund, K. N., G. Aguilera and G. V. Childs (1985). "Quantification of morphological changes in pituitary corticotropes produced by in vivo corticotropin-releasing factor stimulation and adrenalectomy." Endocrinology **116**(1): 439-445.

Whipple, A. O. and V. K. Frantz (1935). "Adenoma of Islet Cells with Hyperinsulinism: A Review." Ann Surg **101**(6): 1299-1335.

White, D., N. Waugh, J. Elliott, J. Lawton, K. Barnard, M. J. Campbell, S. Dixon, S. Heller and R. group (2014). "The Relative Effectiveness of Pumps Over MDI and Structured Education (REPOSE): study protocol for a cluster randomised controlled trial." BMJ Open **4**(9): e006204.

White, N. H., D. A. Skor, P. E. Cryer, L. A. Levandoski, D. M. Bier and J. V. Santiago (1983). "Identification of type I diabetic patients at increased risk for hypoglycemia during intensive therapy." The New England journal of medicine **308**(9): 485-491.

Wilhelm, I., J. Born, B. M. Kudielka, W. Schlotz and S. Wust (2007). "Is the cortisol awakening rise a response to awakening?" Psychoneuroendocrinology **32**(4): 358-366.

Wilson, D. M., R. W. Beck, W. V. Tamborlane, M. J. Dontchev, C. Kollman, P. Chase, L. A. Fox, K. J. Ruedy, E. Tsalikian and S. A. Weinzimer (2007). "The accuracy of the FreeStyle Navigator continuous glucose monitoring system in children with type 1 diabetes." Diabetes care **30**(1): 59-64.

Wilson, D. M., D. Xing, R. W. Beck, J. Block, B. Bode, L. A. Fox, I. Hirsch, C. Kollman, L. Laffel, K. J. Ruedy, M. Steffes and W. V. Tamborlane (2011). "Hemoglobin A1c and mean glucose in patients with type 1 diabetes: analysis of data from the Juvenile Diabetes Research Foundation continuous glucose monitoring randomized trial." Diabetes care **34**(3): 540-544.

Wojcicki, J. M. (1995). "'J'-index. A new proposition of the assessment of current glucose control in diabetic patients." Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et métabolisme **27**(1): 41-42.

Workgroup on Hypoglycemia, A. D. A. (2005). "Defining and reporting hypoglycemia in diabetes: a report from the American Diabetes Association Workgroup on Hypoglycemia." Diabetes Care **28**(5): 1245-1249.

Wright, R. J., D. E. Newby, D. Stirling, C. A. Ludlam, I. A. Macdonald and B. M. Frier (2010). "Effects of acute insulin-induced hypoglycemia on indices of inflammation: putative mechanism for aggravating vascular disease in diabetes." Diabetes care **33**(7): 1591-1597.

Wrosch, C., G. E. Miller, S. Lupien and J. C. Pruessner (2008). "Diurnal cortisol secretion and 2-year changes in older adults' physical symptoms: the moderating roles of negative affect and sleep." Health psychology : official journal of the Division of Health Psychology, American Psychological Association **27**(6): 685-693.

Wrosch, C., G. E. Miller and R. Schulz (2009). "Cortisol secretion and functional disabilities in old age: importance of using adaptive control strategies." Psychosom Med **71**(9): 996-1003.

Wurzburger, M. I., G. M. Prelevic, P. H. Sonksen, L. A. Peric, S. Till and R. W. Morris (1990). "The effects of improved blood glucose on growth hormone and cortisol secretion in insulin-dependent diabetes mellitus." Clinical endocrinology **32**(6): 787-797.

Wust, S., J. Wolf, D. H. Hellhammer, I. Federenko, N. Schommer and C. Kirschbaum (2000). "The cortisol awakening response - normal values and confounds." Noise Health **2**(7): 79-88.

Wynn, P. C., J. P. Harwood, K. J. Catt and G. Aguilera (1988). "Corticotropin-releasing factor (CRF) induces desensitization of the rat pituitary CRF receptor-adenylate cyclase complex." Endocrinology **122**(1): 351-358.

Xu, E., M. Kumar, Y. Zhang, W. Ju, T. Obata, N. Zhang, S. Liu, A. Wendt, S. Deng, Y. Ebina, M. B. Wheeler, M. Braun and Q. Wang (2006). "Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system." Cell metabolism **3**(1): 47-58.

Ye, H., F. He, X. Fei, Y. Lou, S. Wang, R. Yang, Y. Hu and X. Chen (2014). "High-dose atorvastatin reloading before percutaneous coronary intervention increased circulating endothelial progenitor cells and reduced inflammatory cytokine expression during the perioperative period." J Cardiovasc Pharmacol Ther **19**(3): 290-295.

Yeh, H. C., T. T. Brown, N. Maruthur, P. Ranasinghe, Z. Berger, Y. D. Suh, L. M. Wilson, E. B. Haberl, J. Brick, E. B. Bass and S. H. Golden (2012). "Comparative effectiveness and safety of methods of insulin delivery and glucose monitoring for diabetes mellitus: a systematic review and meta-analysis." Ann Intern Med **157**(5): 336-347.

Yoshida-Hiroi, M., M. J. Bradbury, G. Eisenhofer, N. Hiroi, W. W. Vale, G. E. Novotny, H. G. Hartwig, W. A. Scherbaum and S. R. Bornstein (2002). "Chromaffin cell function and structure is impaired in corticotropin-releasing hormone receptor type 1-null mice." Molecular psychiatry **7**(9): 967-974.

Young, J. B., R. M. Rosa and L. Landsberg (1984). "Dissociation of sympathetic nervous system and adrenal medullary responses." The American journal of physiology **247**(1 Pt 1): E35-40.

Yousefzade, G. and A. Nakhaee (2011). "Insulin-induced hypoglycemia and stress oxidative state in healthy people." Acta diabetologica.

Yue, J. T., E. Burdett, D. H. Coy, A. Giacca, S. Efendic and M. Vranic (2012). "Somatostatin receptor type 2 antagonism improves glucagon and corticosterone counterregulatory responses to hypoglycemia in streptozotocin-induced diabetic rats." Diabetes **61**(1): 197-207.

Zammit, N. N., R. E. Warren, I. J. Deary and B. M. Frier (2008). "Delayed recovery of cognitive function following hypoglycemia in adults with type 1 diabetes: effect of impaired awareness of hypoglycemia." Diabetes **57**(3): 732-736.

Zerangue, N., B. Schwappach, Y. N. Jan and L. Y. Jan (1999). "A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels." Neuron **22**(3): 537-548.

Zijlstra, E., T. Heise, L. Nosek, L. Heinemann and S. Heckermann (2013). "Continuous glucose monitoring: quality of hypoglycaemia detection." Diabetes Obes Metab **15**(2): 130-135.

Zong, H., J. M. Ren, L. H. Young, M. Pypaert, J. Mu, M. J. Birnbaum and G. I. Shulman (2002). "AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation." Proc Natl Acad Sci U S A **99**(25): 15983-15987.

Zunz (1929). "Contributions a l'etude des variations physiologiques de la secretion interne du pancreas. relations entre les secretions externe et interne du pancreas." Arch Int Physiol Biochem **31**: 20-44.